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## **LABORATORY PROCEDURES IN PARASITOLOGY**

### **1. Purpose**

This manual has been written as a guide in the collection and examination of various parasitological specimens. Its purpose is two-fold; namely, to serve as a guide for courses of instruction, and secondly, as a reference for technicians in the field.

### **2. Policy**

The field of parasitology is broad and encompasses a wide variety of procedures. This manual is global in application and contains information which may be of value in the diagnosis of parasitic infections which may occur among Armed Forces personnel anywhere in the world.

### **3. Contents**

This manual contains procedures for the collection and examination of specimens such as feces, urine, sputum, blood, cerebrospinal fluid, and tissues and tissue fluids. Methods for preserving, staining, and mounting of the various specimens which may be examined are also included. The relationship between parasitic diseases and their vectors is discussed.

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# INTRODUCTION TO PARASITOLOGY

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## GENERAL

A parasite can be defined as a living thing which lives in or upon another living thing, from which it derives its sustenance. Clinical parasitology is that branch of medical laboratory which deals with the detection of parasitic infections in man. It can be divided into two broad categories; namely, protozoology which is that branch of parasitology which deals with one-celled animals, and helminthology, the branch which deals with infections with helminths or worms. Due to the close inter-relationship between arthropods and the phenomenon of parasitism, certain members of this group must also be considered in any treatise on medical parasitology.

Depending upon location within the human host, various body tissues, fluids, and exudates may yield parasites or stages in their life cycles, upon which a laboratory diagnosis can be established. These include feces, urine, blood, spinal fluid, serum, and various body tissues. From a diagnostic point of view, it has been found most expedient in the present manual to divide the contents into four parts on the basis of the material which is being examined and the parasitic infection for which diagnostic evidence may be sought in those cases where a specific infection is suspected. On these bases the following divisions are set forth:

### INTRODUCTION TO PARASITOLOGY

#### PART ONE—EXAMINATION OF FECES AND URINE

#### PART TWO—EXAMINATION OF BLOOD

#### PART THREE—HISTOLOGIC AND SERADIAGNOSTIC EXAMINATIONS

#### PART FOUR—ARTHROPODS

In this introduction special emphasis has been placed on fundamentals which are prerequisite to the performance of the most routinely requested procedure, namely, fecal examinations. The first requirement is a thorough knowledge of the microscope including

its construction and manipulation in the observation of slide-mounted specimens. Attention has also been given to preparation and handling of reagents, precautions in their use, the care of glassware, and maintenance of records.

In part one the various methods for fecal examinations are first described. In this, as well as in subsequent sections where methodology is dealt with, a broad latitude of choice of procedures is included. This is necessitated by the fact that, depending upon many varying factors, certain procedures may be found superior to others. For example, in selecting the most suitable concentration technique the specific infection suspected must be taken into consideration. In the recovery of the various parasites which occur in stools it is noted that sedimentation by one method or another may be necessary for some species, while in others concentration by flotation techniques may be far superior. The relative advantages and disadvantages of the various procedures are noted for the methods outlined. It is also stressed that when morphological details of suspicious objects cannot be precisely discerned by use of the more rapid temporary staining procedures, permanent stains should be utilized. The method of choice among the various procedures described will depend upon the permanency of the preparation desired and the time which can be spent in preparing the material for study of morphological details. Experience will indicate which techniques yield the best results. In the hands of different technicians, different procedures often give more satisfactory definition of microscopic details in permanent stained mounts.

In part two, parasitic infections which are usually routinely diagnosed by either direct examination or culture of blood or serum are presented. These include malaria, leishmaniasis,

trypanosomiasis, and filariasis. Again, as in part one, numerous variations in techniques are given.

Since histologic and serodiagnostic methods are applicable to organisms ranging from the primitive parasitic one-celled forms to the most highly developed helminths, it seemed more appropriate to discuss them separately than to present them with related material in earlier sections. These methods have, therefore, been elaborated upon separately in part three. Further, these techniques are usually resorted to only in situations where more direct methods fail to resolve a diagnostic problem and their area of applicability is quite restricted compared to earlier sections. Many of the techniques require that the specimen be obtained by the attending physician. The degree of professional proficiency which is needed to obtain and process the specimens relegate them to an area of importance limited to highly qualified personnel. For obvious reasons the arthropods are discussed separately in part four.

This manual is global in application and believed to contain the information which may be required to definitely diagnose essentially all parasitic infections which may occur among personnel anywhere in the world. A comprehensive presentation is necessitated by many factors. The nature of commitments of the Army both in peacetime and in conflicts is such that Army personnel could be exposed to most any of the parasitic diseases which are known to exist. Further, the manual is aimed to meet the requirements of an instructional guide as well as a reference manual for use in our various laboratories in which medical care is provided. Also, it is deemed necessary that it meet the requirements of routine laboratory diagnostic work on the one hand, and epidemiological investigations on the other. Lastly, it is compiled to meet the requirements of the broad range of qualifications and experience ranging from the young, inexperienced technician to the clinical laboratory officer or pathologist.

## THE COMPOUND MICROSCOPE

The compound microscope is one of the most useful and commonly employed items of equipment in the laboratory diagnosis of parasitic infections. It is the most important single piece of equipment in the diagnostic laboratory. Since so much of the work performed in routine examinations requires the use of the microscope, the technician should be thoroughly familiar with the construction, proper use, and care of this delicate instrument.

The microscope is used to magnify and so make visible to the eye very small bodies such as cysts and trophozoites of intestinal parasites, ova of helminths, and parasites in blood, spinal fluid, and other body fluids. A simple microscope is a single magnifying lens. A compound microscope, which is the type used in medical laboratories, consists of a number of lenses so arranged that different degrees of magnification up to 1,000 times can be obtained. The principal parts of the microscope can be divided into the framework, illumination, and magnification systems.

### Parts of the Microscope

Your attention is directed to the various parts of the microscope illustrated and labeled in figure 1. In order to use the microscope effectively, the laboratory worker should be thoroughly familiar with the various parts of this instrument and their functions. The various parts of the microscope and their functions are tabulated in table 1 which follows:

Table 1. Parts of the Compound Microscope and Their Functions

PARTS	FUNCTION
Ocular Lens or Eyepiece	Objects magnified on the microscope stage are observed through the lens of the ocular. Oculars of 5X or 10X magnification are usually standard equipment.
Body Tube	This structure supports the ocular eyepiece and is the portion of the microscope through which light passes from the objectives to the ocular lens.

**Table 1. Parts of the Compound Microscope and Their Functions—Continued**

PARTS	FUNCTION
Revolving Nose-piece	In the microscopes equipped with oil immersion lenses three objectives are mounted on the nosepiece. The desired objective is brought into position by revolving the nosepiece. A slight "click" is felt when the objective comes into proper position.
Objective	These are usually three in number, namely low power, high dry, and oil immersion. The lenses through which light passes initially from the microscopic field are mounted in the objectives.
Sub-Stage Condenser	The lens of the condenser concentrates light reflected from the mirror upward through the microscopic field.
Iris diaphragm Shutter	This lever controls the size of the opening in the sub-stage condenser and, therefore, also assists in controlling the amount of light which passes through the object.
Mirror	The mirror catches and concentrates light and reflects it upward through the condenser and microscopic field to the ocular lens.
Base, Pillar, and Arm	These form the supporting structures for the illumination and magnification systems of the microscope. The base is that portion upon which the microscope rests. The pillar is the upright portion which connects the arm of the instrument with the base. The arm is that portion of the microscope closest to you when a field is under study. It supports the ocular and objectives together with the various parts of the microscope by means of which these are moved to desired positions.
Sub-Stage Adjustment	This knob governs the upward and downward movement of the condenser.
Inclination Joint	This is a pivotal joint which permits the inclination of the microscope to various desired angles.
Stage	This is the square flat surface upon which the slide is placed.
Spring Clip	Spring clips one on each side of the stage clamp the slide securely into position. Mechanical stages are usually substituted. The mechanical stage facilitates systematic, thorough searching of a mount by manipulation of knobs which regulate movements in all directions.

**Table 1. Parts of the Compound Microscope and Their Functions—Continued**

PARTS	FUNCTION
Fine Adjustment	The tube/carrying the ocular and objectives is moved downward toward the slide when this knob is moved in a clockwise direction. (The eye is brought into a position parallel to the microscope stage when the tube is moved toward the stage.)
Coarse Adjust-	First pass the estimated focal plane by rotating the coarse adjustment knob. Then rotate the fine adjustment knob counterclockwise to bring the microscopic field slowly into clearly defined focus.

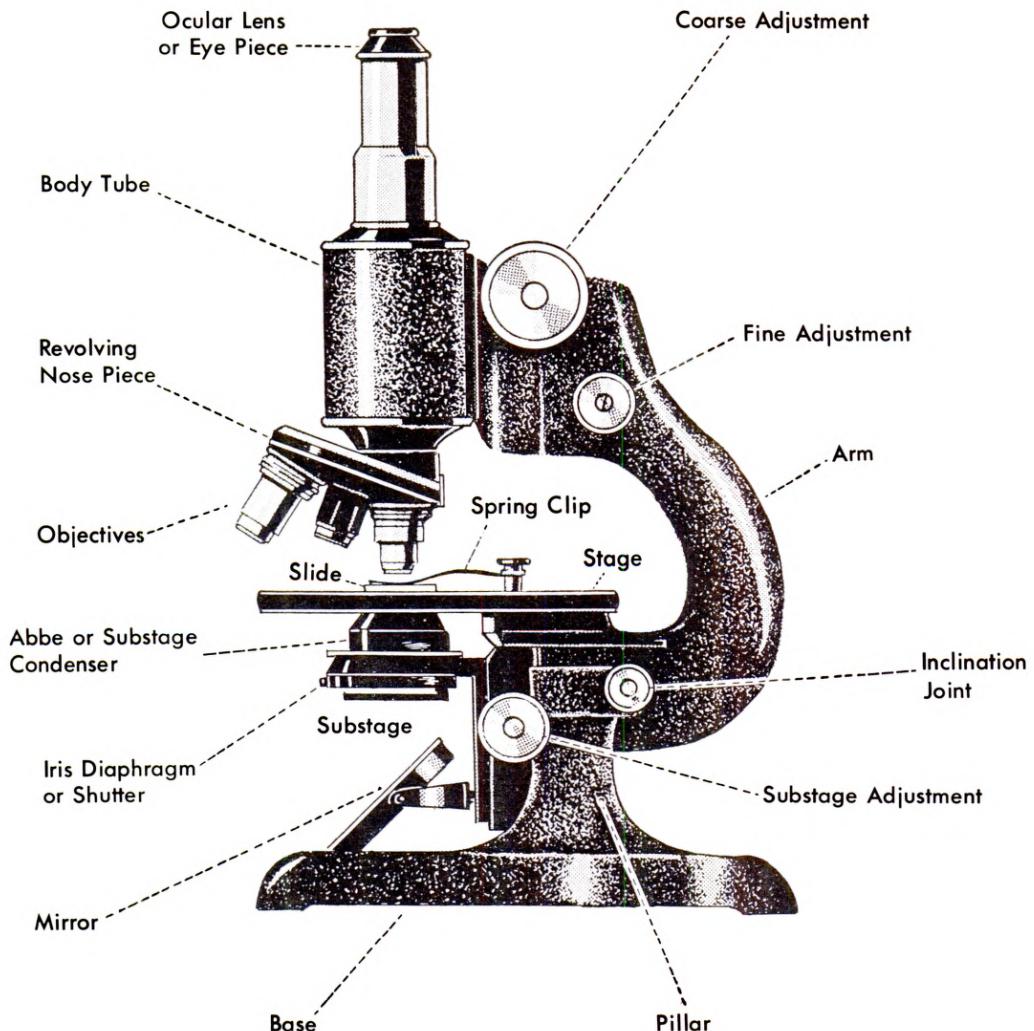
### **Mounting, Placement, and Movement of Object of Study**

To study an object under magnification, material to be examined is first placed on a glass slide and always covered with a coverslip. Material which is not covered may smear the objective.

It is stressed that generally in microscopic work in parasitology, particularly when dealing with wet preparations, microscope stages should not be tilted. This enhances flowing of fluids beneath the coverslip and may result in contamination of objectives and stage when working with material which is potentially infectious.

Mount the preparation to be observed on the microscopic stage and move the area of the slide to be examined to a position directly over the opening in the iris diaphragm which is in the center of the microscope stage. Once the preparation is set on the microscope stage, clamp it into position either in the grip of a mechanical stage or under the stage clips and then move the preparation around until the desired area lies beneath the perpendicular axis of the objective.

Note that as the object is moved on the microscope stage, a movement of the object under observation to the left appears in the ocular as a movement to the right in the field. This is because the image in the ocular is reversed in position to that of the object. Briefly stated, movement to the left will be observed as movement



**Figure 1. A Compound Microscope.**

to the right in the microscopic field, and movement to the right will be observed as movement in the opposite direction. Backward and forward motion is similarly reversed.

Microscopes provided in the medical laboratory are usually equipped with a mechanical stage by means of which the preparation is secured into position and moved about during examination. A mechanical stage is illustrated in figure 2. Many types of examination for parasitic infection necessitate that the entire mount be systematically, carefully, and completely examined. In order to enable thorough examination a mechanical stage is indispensable.

The mechanical stage consists of two arms connected to each other at right angles. Gradu-

ated scales are etched upon these arms and their movement is controlled by manipulation of two adjustment knobs situated in the lower right corner of this stage. To search systematically an entire preparation, move the slide to a position where the objective is directly above the lower left hand corner of the slide. When the microscopic field is viewed through the ocular, this will appear as the upper right hand corner due to image inversion. Using the two knobs, direct movement left, then slowly downward, then right, and repeat these directional movements until the whole preparation has been examined. Figure 3 illustrates the proper directional movements of the slide as it is observed through the eyepiece when the slide is moved about with a mechanical stage.

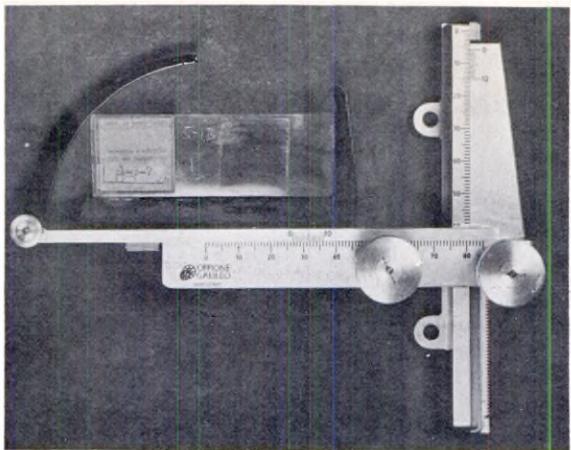


Figure 2. A Mechanical Stage.

## Illumination

Proper illumination of the object under study is an extremely important detail. Improper lighting of the object may lead to inaccurate results and conclusions as well as unnecessary eye strain. Proper lighting depends upon the proper use of the mirror and especially of the iris diaphragm which controls the amount of light which passes upward through the object under observation.

The mirror is double, being flat on one side and concave on the other. The mirror should always be kept free of dust particles and should not be touched with the fingers when its position is adjusted. It should be handled with the frame only. The reversible mirror is provided with a double hinge so that it can be tipped at any angle to obtain the maximum amount of light through the object under study. So far as brightness of illumination is concerned it is immaterial whether the flat or concave side of the mirror is used. However, distracting images such as window bars in the field of vision are more easily eliminated when the concave side of the mirror is used. When using

low power sources of artificial light the concave side of the mirror gives more intense illumination. If the source of constant artificial light is a heavy duty lamp the concave side of the mirror should be used at all times.

The amount of light passing through the specimen is controlled by use of the iris diaphragm in the substage condenser. The size of the opening in the diaphragm is controlled by a lever on the side of the condenser. Moving the lever away from you will cause the opening in the iris diaphragm to become smaller while moving it toward you will cause it to enlarge. Never force the lever of the iris diaphragm to the full limit in either direction. Doing so may damage the delicate leaves of the diaphragm. Generally, when observing liquid preparations such as fresh, wet smears the diaphragm opening is almost completely closed when using low power. Under the high dry objective the diaphragm is generally opened somewhat more to allow more light to pass through the material. When observing stained preparations with the oil immersion objective the iris diaphragm is usually opened wide.

Various factors determine the intensity of illumination which should be used. These depend chiefly upon the individual, the type and intensity of the light source, whether the light source is natural or artificial, and the kind of preparation which is under observation. A general rule is that the higher the degree of magnification, and the greater the density of the object under study, the greater the amount of light required to adequately illuminate the object. A major cause of failure to detect parasites in fecal examinations is failure to maintain proper illumination. Always remember that illumination must be constantly adjusted with each change of magnification and with each object which is observed for morphology.

The distance between the condenser and the stage as well as the size of the opening in the iris diaphragm need constant adjustment to accentuate details of objects observed. Too much light renders objects invisible due to glare while too little light obscures morphologic details. Artificial light is better than daylight, because the intensity of the light re-

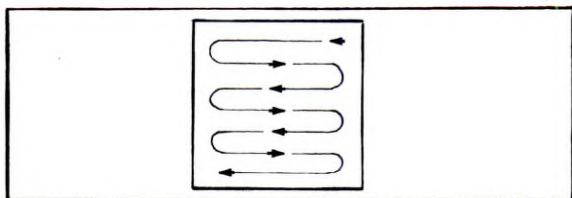


Figure 3. Proper Directional Movement of Slide for Complete Coverage.

mains constant. The size of the opening in the condenser together with its position up or down controls the total light entering the system. The closer the condenser is to the stage the greater the concentration of light, and the farther away the less light which passes upward through the object under observation. For most purposes, artificial light should be filtered through a Corning daylight glass or cobalt glass. For observation of objects under the high dry magnification or oil immersion lenses the condenser should be raised to its maximum level.

When using the oil immersion objective a drop of either mineral oil or oil of cedarwood is placed on the coverslip of the slide. Mineral oil will not become gummy or sticky. While oil of cedarwood tends to become more gummy it does give slightly better optical results. To remove oil from slides and objectives, xylol is used and it is equally satisfactory for the removal of either mineral oil or cedarwood oil.

Improper illumination is indicated when:

1. Dark points or shadows appear in the field.
2. The outline of an object is bright on one side and dark on the other.
3. When the object appears to be in a glare of light.

These can usually be corrected either by suitably adjusting the position of the mirror or reducing the amount of light by adjusting the size of the opening in the iris diaphragm.

## Magnification

Microscopes in general use in medical laboratories are provided with three objectives, namely, 1.9 mm, 4 mm, and 16 mm respectively. Microscopes are usually provided with 5X and 10X ocular eyepieces. Multiplying the power of the ocular by the power of the objective gives the degree of magnification of the object under observation. The following is a table showing the ocular magnification, the millimeter length of the objective, and its magnification power, and the total diameter increase obtained using oculars and objectives of the powers shown.

**Table 2. Oculars, Objectives, and Magnification Powers**

OCULAR	OBJECTIVE	MAGNIFICATION
5X	16 mm (10X)	50 diameters
10X	16 mm (10X)	100 diameters
5X	4 mm (43X)	215 diameters
10X	4 mm (43X)	430 diameters
5X	1.9 mm (97X)	485 diameters
10X	1.9 mm (97X)	970 diameters

The laboratory worker should practice identifying objects such as cysts, trophozoites, and ova of helminths and the like under low power magnification turning either to high dry or oil immersion for more careful study of suspicious objects. Always remember that the lowest magnification which will give detailed definition is the desired magnification to be utilized. The greater the magnification of the object, the greater the distortion and the greater the requirement for careful manipulation to bring the object into view.

## Focusing

Focusing can be defined as the adjustment of the relation between the optical system and the object so that a clear image is obtained. Several important rules to be observed in focusing objects under observation in the microscope are as follows:

1. After the preparation to be observed is mounted on the stage, the objective to be used is turned in line with the eyepiece.
2. Movement of the objective is accomplished by revolving the nosepiece. The nosepiece is provided in order to enable rapid, convenient substitution of one objective for another. This change is effected by grasping two of the objectives between the thumb and forefinger of the right hand and rotating them until the desired objective is brought into line with the axis of the body tube. It is very important that exact alignment be obtained. The correct setting is indicated by a slight "click" as the objective comes into position.
3. Whenever the nosepiece is revolved its movements should be observed to make certain that the objectives are not racked

into the preparation. Some microscopes are not par-focal, that is, objects in focus under low power will not be in focus when the nosepiece is rotated to a higher power of magnification. It may, therefore, be necessary to re-focus when changing to higher magnification to prevent contact between the specimen and the lens as the objectives are rotated. In microscopes which are par-focal it is possible to swing other objectives into place without touching the coarse adjustment and with only slight turn of the fine adjustment knob required to restore perfect focusing.

4. Before commencing to bring the object into focus, lower the head until the eye is on a level with the preparation.
5. Then, using the coarse adjustment, lower the objective until it is below the point at which the object would normally be expected to come into view. Then, using the fine adjustment and at the same time looking through the ocular, raise the objective very slowly, until the field comes into view. Further adjust to the best image using only the fine adjustment.
6. In focusing upward with the fine adjustment, the object will first appear in faint outlines and indistinctly, then gradually more distinctly, and finally, sharply defined. If the adjustment goes beyond the point of sharp definition, it will gradually become more dim, in which case return to the point of greatest clarity.
7. If difficulty is experienced in bringing an object into the field of view, this can be corrected either by using a lower powered eyepiece or by substituting a lower powered objective to find the desired field. In doing this, a larger field is obtained in which the object may be more easily found. After the object is centered on lower magnification, switch to a higher power objective for observation of details.
8. NEVER MOVE AN OBJECTIVE DOWNWARD TO FIND A FIELD

WHILE LOOKING THROUGH THE EYEPIECE. WHEN THE OBJECTIVE IS MOVED DOWNWARD ALWAYS OBSERVE THE DOWNWARD MOTION WITH THE EYE HELD LEVEL WITH THE MICROSCOPE STAGE. See figure 4. Failure to observe these precautions may result in damage to the lens of the objective or the preparation under study.

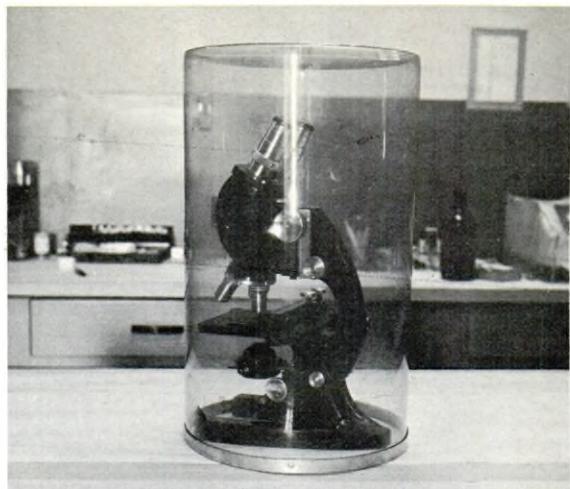
### Care of the Compound Microscope

The microscope is an instrument of precision with many delicate parts which MUST be handled with the utmost care. The following precautions should always be observed in the care of the microscope:

1. The microscope should always be covered when not in use. See figure 4.
2. Care should be taken to prevent all parts of the microscope from coming into contact with acids, alkalies, chloroform, alcohol, or other substances which corrode metal or dissolve the cementing substance by means of which the lenses are secured into the objectives and oculars.
3. Only lens papers should be used in cleaning the ocular or objective lenses, see figure 4. Do not wipe lenses with gauze or fibrous cloth.
4. Never touch the lenses in the ocular, objective, or substage condenser with the fingers. Even slight perspiration of otherwise clean hands may damage the lens glass which is soft and easily marred.
5. The only cleaning agent which should be used in cleaning lenses or removing oil from objectives is xylol.
6. When sewing machine oil is used to lubricate moving parts of the microscope all excess should be wiped off to prevent the collection of grit and dust.
7. The microscope should be protected against direct sun and moisture.
8. In very warm, humid climates microscopes should be stored in dry cabinets



**Downward Focusing is Accomplished Under Observation**



**The Microscope is Covered When Not in Use**



**Lenses Are Cleaned With Xylol Soaked Lens Paper**



**Use Both Hands to Support a Microscope**

**Figure 4. Care and Use of the Microscope.**

when not in use. Such cabinets should be reasonably airtight, equipped with a light bulb to supply heat, and several cloth bags containing a hygroscopic salt such as calcium chloride should be placed in the chamber to absorb moisture. In warm, humid climates the lenses of unprotected microscopes may be attacked by certain fungi which etch glass and ruin the lenses.

9. After use, always turn the nosepiece to a position which brings the low power

objective into direct line with the opening in the substage condenser. If this precaution is not taken, the longer higher powered objectives may rack into the condenser lens.

10. In moving the microscope from one place to another, always grasp the arm of the instrument firmly with the right hand and support the base with the left hand. The proper method of carrying a microscope is illustrated in figure 4.

11. The entire microscope should be cleaned frequently to remove dust, finger marks, oil, grease, and remnants of specimens. All parts of the microscope should be kept scrupulously clean at all times.
12. Never tamper with any of the parts of the microscope. If this instrument does not seem to be functioning properly immediately call the matter to the attention of the laboratory officer.

## LABORATORY RECORDS AND REPORTS

### Purpose

Reports of laboratory parasitological examinations serve three principal purposes; they furnish useful information to the clinician for diagnosis, serve as a guide for treatment and follow-up to determine effectiveness of treatment, and may under certain conditions yield information which is of value in epidemiological studies and furnish basic data for correction of deficiencies in environmental sanitation programs.

### Initiation of Requests

Requests for laboratory examinations on patients are initiated by the attending physician. SF 514G, "Feces," properly filled out and signed is submitted to the laboratory in duplicate with the parasitological specimen. A copy of SF 514G with the completed laboratory report is shown in figure 5. When the clinician desires special tests or that certain specific observations be made he will usually make an entry to this effect in the remarks section on SF 514G or will make an entry under the item "other tests or examinations." These may include such entries as "suspected amoebic dysentery," "check for *Taenia saginata*—anthelminthics administered 3 June 1958," "check for roundworm infection," etc.

### Log Book Record

A log book should be maintained in each section of the laboratory. This is a chronological record of specimens received and results of examinations. When a specimen is brought into the laboratory it is assigned a number and posted in the log book. Prior to submitting the laboratory report indicating findings in an examination, results are posted in the daily

log. Log records are conveniently maintained in bound lined ledgers with column headings written across two facing pages. Suggested column headings for entry in the daily log include date specimen submitted, specimen number (assigned by section chronologically for each day's work), name of patient, patient's status (ward number, if hospitalized, outpatient, or other), requesting physician, remarks (copy from remarks section of SF 514G), findings, and examiner's initials. The daily log serves as a very useful record particularly when specimens require examination procedures necessitating processing over a period of several days.

### Completion of Entries and Disposition of Report Forms

Upon completion of the appropriate entries in the laboratory report slips, (SF 514 series) the original on hospitalized personnel or outpatients is returned to the office of origin where it is filed with the patient's clinical record. These reports having epidemiologic or sanitary reference are forwarded to the preventive medicine officer, veterinarian, sanitary engineer, or other department concerned. Duplicate copies are retained in the laboratory office files. These serve as a permanent file record for reference purposes and reflect complete data on all examinations performed. Reports should be neat, concise, accurate and complete. Whenever possible every effort should be made to examine a specimen and submit the report on the date the specimen is received for examination. Entries on the laboratory reports are generally made by the technician or officer making the examination. All laboratory reports must be signed by the laboratory officer.

REGISTER OR UNIT NO.		WARD NO.	<input checked="" type="checkbox"/> BED PATIENT <input type="checkbox"/> AMBULATORY
872		3	
REQUESTED BY AND DATE		DATE AND TIME COLLECTED	
Dr Ainsworth 8/14/58		0730 14 Aug	
CLINICAL DATA			
Motile trophozoites with red blood cell inclusions observed			
PATIENT'S LAST NAME—FIRST NAME—MIDDLE NAME			
MacMullen, William Louis			
APPEARANCE Chalky			
CONSISTENCY Fluid			
BLOOD—GROSS Bloody mucus			
OCCULT			
PUS			
MUCUS			
BILE			
OVA AND PARASITES			
REMARKS			
Suspected amoebic dysentery			
DATE OF REPORT		SIGNATURE (Specify Lab. if not part of requesting facility)	
8/14/58		B.O. Adams, MC	
NAME OF MEDICAL FACILITY			
7280th USAF HOSPITAL			
Standard Form 514-G—Rev. June 1959. * U. S. GOVERNMENT PRINTING OFFICE: 1959 J-530187 Bureau of the Budget Circular A-32			
FECES			

Figure 5. SF514G, Request for Examination for Ova and Parasites.

## LABORATORY REAGENTS

### Preparation of Reagents

Various stains and solutions are utilized in routine parasitological examinations. These must be prepared with the utmost care and precisely according to formulations. Detailed directions for the preparation of all reagents which are required for performing procedures outlined throughout this manual are contained in appendix 1. Careful attention should be given to precise measurements, order in which reagents are added, control of temperature where indicated, filtration and aging. Particular attention must be given to storage of reagents particularly with reference to requirements for refrigeration, incubation, and protection from intense light.

### Labeling Reagent Containers

Proper labeling of reagents is an extremely important detail. Labels should be complete, securely attached, and neatly and legibly written or preferably typewritten. Items recorded on the label should include (in the order listed) : Name of the reagent, exact formula including all constituents and quantities utilized,

date of preparation, initials of the individual who prepared the reagent, and expiration date if the solution deteriorates with age. In figure 6 note the proper method for labeling a reagent container. Labels should be protected against damage by water or other fluids by covering with a protective coating of warm paraffin, collodion or shellac painted over the surface of the label.

### Precautions in the Handling of Reagents

There are various precautions which must be taken in handling reagents in the parasitological laboratory. Among the most important are the following:

- Once a portion of a reagent has been removed from the original container it should never be poured back. Doing so may contaminate the remaining unused portion.
- Reagents are preferably stored in alphabetical order on shelving protected from dust, moisture, and direct sun.
- Never use a reagent which cannot be clearly identified from the label on the

- container. Discard all reagents which cannot be accurately identified. See figure 6.
4. Always read the label before dispensing a reagent.
  5. Working with newly prepared reagents, especially stains, ascertain whether desired results are being obtained. Unsatisfactory solutions should be discarded and replaced.
  6. All mixing containers, stirring rods, and containers used for storage of reagents should be chemically cleaned prior to use.
  7. During mixing and preparation as well as in storage it is good practice to avoid contact of reagents with metals. Many reagents contain substances which will react chemically with metal producing changes which will render them unusable for laboratory work.
  8. Do not allow inexperienced personnel to prepare reagents without close supervision.

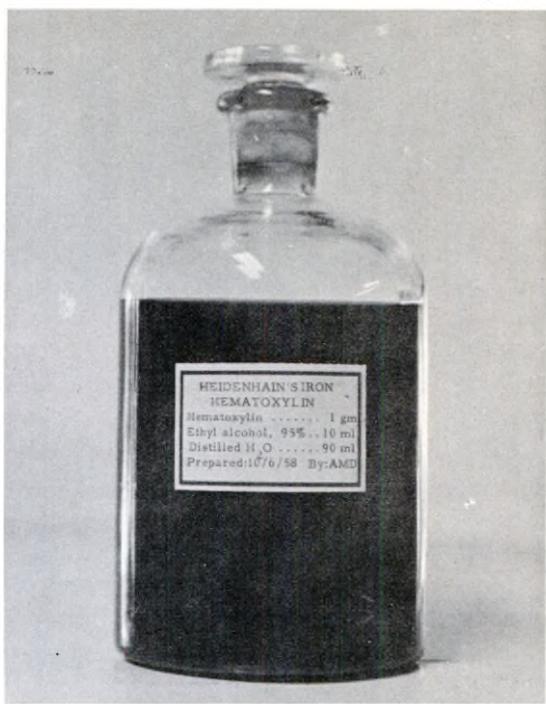


Figure 6. Properly Labeled Reagent Container.

## THE CARE OF LABORATORY GLASSWARE

### Mechanical Cleaning

Cleaning of glassware is greatly facilitated if it is immersed in a solution immediately after use to prevent drying and encrustment with adhering debris. For ordinary laboratory use either new or used glassware can be adequately cleansed by thorough scrubbing in hot, soapy water. Following thorough cleansing items should be passed through several rinses of hot, tap water, with a final rinse in two changes of hot distilled water. Clean glassware should be placed upon freshly laundered Turkish towels, and spread out in a single layer, and air dried in a dust-free room. In laboratories equipped with dry heat ovens, glassware can be dried by heating for 30 minutes at 150° C. After thorough drying place cleaned items in suitable containers for protection from dust during storage.

### Chemical Cleaning

If mechanical cleaning cannot adequately clean glassware, or if it is to be used in procedures requiring removal of all traces of foreign substances it must be chemically cleaned. The most suitable solution for initial cleaning of glassware by this procedure is a mixture of sulphuric acid and sodium dichromate. For method the preparation of this solution, check appendix 1.

In the rinse water much more efficient cleansing can be obtained if wetting agents are added. The efficiency of these wetting agents is due to their ability to reduce surface tension, thereby causing water containing them to wet objects much more thoroughly and more quickly. Following several rinses to remove all traces of the cleaning solution it is desirable to pass glassware again through a hot soapy solution and include a wetting agent in the

solution if available. Following the last cleaning glassware is again rinsed several times in tap water, then in distilled water, and finally in 95 percent ethyl alcohol.

#### **Special Precautions for Cleaning Pipettes, Test Tubes, Slides and Coverslips**

Immediately after use pipettes should be submerged in water. Jars in which pipettes are placed should be deep enough to immerse them to their full length and the bottom should be lined with cotton to protect the delicate tips against breakage. If they have been used in handling of infectious material, they should be placed in a 5 percent solution of phenol for 30 minutes or more. Following immersion in this solution rinse the pipettes thoroughly under the full force of the water tap. Next immerse them in cleaning solution for about 12 hours and clean in the manner outlined in the section above. In cleaning test tubes if they contain culture media in which infectious organisms have been cultured they should first be autoclaved at 15 pounds pressure for 15 minutes.

After autoclaving, test tubes should be

soaked in hot water to remove adhering media and should then be scrubbed thoroughly with a test tube brush in a hot solution of soap and water. If media still adheres to the test tubes it may be necessary to boil them in a hot soapy solution for 30 minutes or longer. Following thorough cleaning in hot soapy water the tubes should be immersed in tap water overnight, rinsed with distilled water several times, and dried the following day. Inspect the tubes, removing any which are not perfectly clean and soak them in acid-chromate solution.

Both new and used slides should first be boiled in soap suds containing 5 percent sodium bicarbonate. Following boiling scrub with soap and water followed by several rinses in tap water. Immerse in acid-chromate cleaning solution overnight. Remove from cleaning solution and rinse for 5 minutes under hot running tap water. Place cleaned slides in a large beaker and cover with fresh 95 percent ethyl alcohol. Polish individually with lint-free cloth (not gauze). Pass slides quickly through the flame of a Bunsen burner and store in closed containers to protect against dust.

# PART ONE

## EXAMINATION OF FECES AND URINE

### Chapter 1

#### METHODS FOR EXAMINATION OF FECES AND URINE

---

The intestinal canal is the normal habitat and escape route of many organisms which parasitize man. Objects of significance which may be revealed in fecal examinations include helminth ova, larvae, and adults; protozoan trophozoites and cysts; discharged body cells such as macrophages, neutrophiles, eosinophiles, and erythrocytes; Charcot-Leydon crystals which are sometimes associated with certain parasitic infections; and various fungi and yeasts including *Blastocystis hominis* which may be confused with cysts and ova. Only natural or saline purged specimens are suitable for diagnostic work. Specimens should not be taken following administration of oil, bismuth, or barium.

There are many variations in the procedures which may be followed in fecal examinations. These depend upon a number of factors such as time elapsing between passage and examination, the infestation suspected, the amount of time which can be spent in examination, materials and equipment available, and the technical qualifications of persons performing the examinations. Medical laboratories generally establish the basic procedures which will be carried out routinely. These usually include simple initial rapid screening tests which serve to indicate the more refined techniques which may need to be carried out to complete the examination. A recommended routine is outlined on page 1-26.

Fecal specimens should be collected in disposable cardboard containers. Pint-sized, paraffined ice cream boxes make excellent specimen receptacles since they are large and assure

less likelihood of reaching the laboratory in a messy condition. They are cheap and can be discarded after use. Glass containers are definitely inferior since they are messier to handle, require the unpleasant task of cleaning after each use, and introduce the possibility of contamination from specimens previously submitted in them. Proper instructions should be given to the patient to avoid contamination of the specimen with urine or water,<sup>1</sup> together with such other instructions as experience may indicate are necessary.

As soon as a specimen arrives in the laboratory its consistency and time of passage should be noted and recorded on SF 514G. Also record whether the specimen is watery, loose, soft, or formed. Macroscopic examination should also be made for adult organisms, otherwise the proglottids of tapeworms and adult nematodes may be overlooked. Where the purpose of the examination is to determine the presence of adult helminths (for example, following administration of anthelmintics) several procedures may be followed. A small portion of the specimen should be retained for microscopic examination. The remainder of the specimen may be comminuted in water and transferred to a dark-bottomed pan<sup>2</sup> and searched for adults. A hand lens is helpful for more detailed examination of objects suggestive of small nematodes. Another method consists of comminuting the entire specimen

<sup>1</sup> Urine distorts ova and cysts. Contamination with water may introduce free living forms which could be confused with intestinal parasites.

<sup>2</sup> The bottom of a pan can be painted with black enamel for this purpose.

in water and passing it through a #30 to #60 sieve, and then searching for adults on the sieve with a hand lens.

In general, the sooner a specimen can be examined following passage the more reliable the results will be. If several specimens are received in the laboratory at the same time, examine the watery, loose, and soft stools before examining the formed ones. Fluid stools may contain trophic forms which will soon perish. There should be no undue delay in examining formed stools if delay can be avoided inasmuch as motile organisms may occur on their outer surfaces. If for any reason formed stools must be retained in the laboratory for an appreciable length of time before examination, they may be stored in the refrigerator at ordinary refrigerator temperatures for periods up to several days. Formed stools are likely to contain cysts and eggs primarily and these will be well preserved by refrigeration. Fluid stools received in fresh condition may be stored in the incubator. This storage should not exceed 30 minutes because increased bacterial multiplication quickly destroys motile organisms.

In numerous situations it may be necessary to forward fecal specimens to a laboratory through the mail. Smaller laboratories, particularly those at dispensaries, may either desire or be required to forward specimens upon which certain diagnoses have been made to larger central laboratories for confirmation. In survey work it often becomes necessary to mail fecal specimens. Certain postal regulations must be observed. These require the use of a double container in which the receptacle containing the specimen is placed. The specimen can be introduced into a round one-ounce sputum shell vial and corked; however, the screw-cap bottles are preferable and should be used when available. The vial or bottle in turn is suspended in cotton packing in a metal, screw-cap container and the latter is shipped in a standard cardboard and metal, screw-top mailing tube. The above method of packing meets all postal requirements. The vial containing the specimen should not be filled to capacity because rapid fermentation, especially in hot weather, will result in gas production

and the cork may blow off. Do not add urine or water to the specimen. Similarly, disinfectants and preservatives should not be added inasmuch as they interfere with flotation techniques. A slip with necessary data concerning the source of the specimen together with any special instructions should be wrapped around the inner metal container. The specimen should be mailed on the same day it is passed.

If adult worms or parts thereof are forwarded for identification, they should be washed free of all debris and placed in 70 percent alcohol or 10 percent formalin for shipment. In the section which follows, the polyvinyl alcohol (PVA) method for preservation and shipment of fecal specimens described is particularly applicable to shipment of specimens known or suspected to contain trophic amoebae.

Inaccurate or incomplete identification of specimens can lead to grave error in the laboratory. Where appreciable numbers of specimens are received a routine procedure which has been found very workable is to assign a number to each specimen for each day's work. This number is affixed to each slide, tube, or other device to which the specimen is transferred during examination.

Standard-sized slides tend to increase the possibility of contamination. Larger slides,  $1\frac{1}{2} \times 3$  inches or  $2 \times 3$  inches are preferable for temporary mounts. Toothpicks, applicator sticks or other implements used to transfer or mix fecal suspensions should be used only once. All objects which come into contact with fecal specimens, i.e., applicators, slides, coverslips, and the like, should be placed immediately in a solution of 5 percent phenol or other suitable disinfectant after use. Always avoid the possibility of infecting yourself or others by carefully handling and disposing of all objects with which fecal specimens come in contact.

In the preparation of temporary mounts such as saline, iodine, or vital stained preparations, use only a small amount of feces. The total quantity of fluid under the coverslip should not exceed about three drops. It is particularly important that inexperienced technicians pre-

pare rather thin preparations. With increased experience thicker preparations can be used effectively. Always coverslip temporary preparations to avoid the possibility of contaminating microscope objectives. In applying cover-slips do not exert excessive pressure. Doing so will force the fluid suspension out and also tends to crush ova which may be present. Certain precautions must be taken in the handling of fecal suspensions which are centrifuged at any stage in their processing. Make certain that the centrifuge is properly balanced and that the mouths of the tubes are plugged in such a manner that the plugs will not be thrown down into the tubes. Centrifuges contaminated as a result of breakage of tubes or splattering due to entry of plugs into their contents are difficult to disinfect properly.

When the presence of *E. histolytica* is suspected but stools are negative, positive specimens may be obtainable from ulcerative lesions in the rectum or sigmoid. These specimens are obtained through a proctoscope or sigmoid-

scope by the physician. Arrangements should be made to have laboratory personnel present to receive the specimen and take it to the laboratory for immediate examination.

Examination of material from a single stool specimen undoubtedly results in the missing of many parasitic infections. With many parasites the passage of stages of the life cycle in the fecal mass is irregular. It can only be concluded from all available evidence in the numerous studies on rate of parasite detection that a minimum of three stools should be examined on each patient preferably on alternate days. These should be followed by examination of a purged stool if prior results are negative. The latter procedure is particularly applicable to fecal examinations in cases of suspected infection with *Endamoeba histolytica*. However, in some cases cyst passage is so sporadic that even three consecutive examinations may fail to reveal infection with this organism.

## PREPARATION AND EXAMINATION OF TEMPORARY MOUNTS

Following macroscopic examination, the next step in the routine examination of a fecal specimen should be the preparation of two temporary mounts on the same slide. These consist of a saline suspension and an iodine stained suspension on opposite ends of the slide. The saline suspension is prepared first, and then with the same applicator stick a portion of the specimen is transferred to the iodine stain. Fecal samples should be taken from several portions of soft and formed stools. The more heterogeneous the stool specimen, the more samples should be taken. If flecks of mucus or blood occur on the outside surface of formed stools these should be selected for examination. When trophozoites are suspected in fresh preparations various techniques may be employed to keep the specimen warm during examination. Both the slides and saline may be placed in a 37° C. incubator prior to preparation of the mount. Slides should not be warmed on microscope lamps inasmuch as temperatures attained are difficult to control or ascertain. Commercial type warm stages are excellent but not ab-

solutely essential for examination for trophic forms. Placing a heated penny on the saline suspension end of the slide helps to keep the preparation warm during examination. It should also be pointed out that in saline preparations time must be allowed for the trophic amoebae to settle down and commence their amoeboid activities. The trophic amoebae will be rounded up and immotile if the preparation is examined too soon. Some workers prefer to make the saline mount, apply a coverslip ringed with petrolatum, and warm the preparation in the incubator for about 5 minutes before examining for motile organisms.

Temporary mounts should be searched systematically and the entire preparation should be examined. It is good practice to utilize low power, switching to the high dry objective for a more careful examination of any objects suggestive of parasites. If no organisms can be found with the low power objective a short final search should be made under high dry magnification to avoid overlooking smaller organisms which might otherwise be missed.

The search should be orderly, starting at the top right hand corner (because of inversion of the image this is actually the lower left) and moving back and forth across the preparation until the entire area under the coverslip has been searched.

One of the major causes of failure to detect parasites in feces is the failure to maintain proper illumination. Illumination must be constantly adjusted with each change of magnification and with each object which is observed for morphology. The distance between condenser and stage as well as the size of the opening in the iris diaphragm need constant adjustment to accentuate details of objects observed. Too much light renders objects invisible while too little light obscures morphologic details.

### **Direct Wet Smear**

It cannot be emphasized too strongly that the *very first step* in the microscopic examination of a fecal specimen for parasites should be the preparation and examination of a direct wet smear. This is the easiest direct method for screening specimens submitted to the laboratory for examination. The unstained portion of the mount will contain the trophic forms, when present, while the iodine stained portion of the mount facilitates detection of organisms in the cystic stage and allows for detailed study of their internal morphology. Ova can be readily observed in either the iodine-stained or saline portion of the preparations.

#### *Materials and Equipment:*

- Medicine dropper
- Physiological saline (54)
- Coverslips
- Slides
- Iodine solution (39)
- Applicator sticks

#### *Technique:*

1. With a medicine dropper place a drop of physiologic saline in the center of one end of the slide and a drop of any of the various standard iodine stains in the center of the opposite end.

2. With a wooden applicator stick, select small portions of feces from various parts of the fecal mass. If the stool contains blood or mucus, these portions should be selected.
3. Mix and spread a small quantity of the specimen evenly with a wooden applicator on the saline portion of the preparation. Similarly mix and spread a small portion of the material on the iodine side of the preparation. Proper density of the smears is indicated when newspaper print can barely be read through the preparation.
4. Apply coverslips to the two smears and examine under the low power objective switching to high dry for observation of details of suspicious objects. Commencing at one corner of each temporary mount, both of the preparations should be systematically and completely searched.

### **Iodine Stains**

Methods of preparation of iodine stains and the duration of their effectiveness after preparation are enumerated elsewhere. The necessity of utilizing stain of proper age, and stain which will give proper intensity of contrast cannot be overemphasized. Stain which is too concentrated is absorbed so rapidly that the entire cyst assumes a uniform dark brown hue. Stains which are too low in concentration are not absorbed sufficiently and the cysts tend to blend in with their surroundings rendering them invisible against the similarly colored lemon-yellow background. In old stains there is a tendency for the iodine to become sublimated. Also, if the stain is left on the slide too long before the feces is mixed with it, uneven staining, as well as over-staining, is likely to result.

### **Vital Stains**

The nuclei of trophic amoebae are indistinct or entirely invisible in saline preparations. Since solutions containing iodine destroy trophic organisms immediately they are unsatisfactory for demonstrating living organisms.

These difficulties have been overcome by the development of suitable vital stains, two of which will be described here.

#### **Staining with Quensel's Solution:**

##### *Materials and Equipment:*

Medicine dropper

Quensel's solution (57)

Slides

Coverslips

Applicator sticks

##### *Technique:*

1. With a medicine dropper transfer a drop of Quensel's solution to the center of a glass slide.
2. Pick up a small amount of the fecal specimen with an applicator stick. Transfer it to the drop of stain on the slide and mix thoroughly.
3. Coverslip and let the preparation stand for 10-20 minutes to allow permeation of the organisms by the staining solution. Slides should be placed in a 37° C. incubator during this interval.
4. Following staining examine the preparation under high dry magnification. Cytoplasm will be stained a pale blue and the nuclei a deeper shade of blue. Stained nuclei present the same morphological characteristics as in permanent hematoxylin preparations. The nuclei of *Dientamoeba fragilis* do not stain well but the presence of 1 or 2 nuclei is clearly discernible. Ciliates, flagellates, and cysts do not stain. After 1-2 hours the motile organisms become overstained and nuclear morphology can no longer be discerned.

#### **Staining with Velat, Weinstein, and Otto Solution:**

##### *Materials and Equipment:*

Medicine dropper

Velat et al's. solution (70)

Slides

Coverslips

Applicator sticks

##### *Technique:*

1. With a medicine dropper transfer one or two drops of the staining solution to the center of a glass slide.
2. With an applicator stick pick up a small amount of the fecal specimen, transfer it to the stain and mix thoroughly.
3. Let stand for 5 minutes in a 37° C. incubator and examine. Critical staining time is about 5 minutes at pH range of 4.6-4.8. At higher pH ranges staining time is from 5-15 minutes. There is less tendency for organisms to overstain at higher pH range.
4. The nuclei and karyosome bodies of all the amoebae stain purple-black, and the cytoplasm a light purple.
5. Flagellates usually become immobilized and tend to swell to some extent but characteristic structures of the various species are usually easily observed. The chromatin of the nucleus of *Chilomastix mesnili* stains a light purple. The nuclear membrane, fibrils, and flagella of *Giardia lamblia* stain a purple-black while the cytoplasm stains a light purple-pink color.

#### **Merthiolate-Iodine-Formalin Staining of Protozoan Cysts (MIF) in Fresh Fecal Specimens (Sapero, Lawless, and Strome)**

This is a simple rapid technique useful for accentuating morphology of cysts. Added to fresh fecal specimens it also serves as a preservative in which specimens can be shipped or stored. The staining reaction with this solution occurs in two phases, an initial iodine phase in which cysts are stained yellow-brown followed by an eosin phase in which the cytoplasm of cysts takes on a pinkish hue. Nuclei stain dark red to jet black. The nuclear elements of all human protozoan cysts are well defined.

##### *Materials and Equipment:*

Medicine dropper

Distilled water

Slides  
Coverslips  
MIF solution (prepare a fresh staining solution once each week) (51)  
Applicator sticks

*Technique:*

1. With a medicine dropper place a drop of distilled water on a slide and add an equal amount of the MIF stain.
2. To this mixture add a small portion of the fecal specimen and mix thoroughly.
3. Coverslip and allow the preparation to stand for a few minutes to permit permeation of cysts by the staining solution.
4. Examine under low power and confirm findings under high dry magnification.

## Concentration Techniques

### Zinc Sulfate Centrifugal Flotation (Faust).

This is a very efficient technique for the detection of helminth ova, larvae, and adults of some of the smaller nematodes. It is also useful for the recovery of protozoan cysts. Fluke and tapeworm eggs bearing opercula, and the nonoperculate ova of the schistosome flukes are not generally recovered by this method. The method is economical and rapid and is recommended as a routine procedure in medical laboratories.

*Materials and Equipment:*

Applicator sticks  
Test tube, 15 ml  
Funnel, 50 mm  
Gauze  
Wassermann tube  
Zinc sulfate solution, specific gravity 1.18-1.20 (75)  
Test tube rack  
Slides  
Coverslips  
Loop, 5 mm attached to holder (Plane of the loop bent at right angle to the wire)  
Iodine solution (39)

*Technique:*

1. Using two applicator sticks, remove a sample of feces about the size of

a pecan. Place the fecal specimen in a test tube containing about 10 ml of tap water and mix very thoroughly with the applicators.

2. Strain this suspension through a 50 mm funnel lined with two layers of wet gauze into a Wassermann tube filling it two-thirds full.
3. Centrifuge this preparation for 1 minute at about 2,500 rpm. This step throws ova, cysts, and other heavier particles to the bottom of the tube, while oils and lighter debris remain in the supernatant.
4. Pour off the supernate fluid and add a small amount of tap water to the tube and break up the sediment with an applicator stick. After breaking up the sediment, fill the tube two-thirds full with tap water once more.
5. Again centrifuge at 2,500 rpm for 1 minute repeating this washing procedure until the supernatant is relatively clear. (Usually a total of 3 washings suffices.)
6. Decant the supernate and add sufficient zinc sulfate solution to fill the tube half full. With an applicator stick break up the packed sediment very thoroughly, then add zinc sulfate solution to fill the tube to within an inch of the top.
7. Centrifuge again for 1 minute at 2,500 rpm. The latter step causes certain parasite ova, larvae, cysts, and some adults to float to the top. Heavier fecal debris will be thrown to the bottom.
8. Carefully remove the tube from the centrifuge and without agitating the contents place it in vertical position in a rack.
9. Wait 1 or 2 minutes, then touch a wire loop of approximately 5 mm diameter to the surface film removing several loopfuls in this manner to a glass slide. It is important that the loop be parallel

- to the surface film and the holder held perpendicular to the film.
10. Add a drop of iodine solution, mix thoroughly, and coverslip. Systematically search the entire mount with the low power objective switching to high dry for observation of details of suspicious objects.

**Modified Zinc Sulfate Centrifugal Flotation.** This modification of Faust's original method saves time and equipment since the straining of the fecal suspension through gauze is omitted, and washing by centrifugation is performed only once instead of 2 or 3 times. Removal of the flotage by superimposed cover-slip also simplifies the technique.

*Materials and Equipment:*

Applicator sticks  
Wassermann tube  
Zinc sulfate solution, specific gravity 1.20 (75)  
Test tube rack  
Slides  
Iodine solution (39)  
Coverslips

*Technique:*

1. Using 2 applicator sticks, remove a sample of feces about 2 ml in volume. Place the fecal specimen in a Wassermann tube one-half filled with tap water and comminute very thoroughly, breaking up all obvious particles.
2. Centrifuge this preparation for 1 minute at approximately 2,500 rpm.
3. Pour off the supernatant fluid and repeat this washing only if the stool is extremely oily. Add enough zinc sulfate solution to fill the tube one-half full and then with an applicator stick break up the packed sediment very thoroughly.
4. Add zinc sulfate to fill the tube to within an inch of top and centrifuge again at 2,500 rpm for 1 minute.
5. Without shaking or spilling, carefully place the tube in vertical posi-

tion in a rack and then slowly add zinc sulfate solution until the tube is brimful.

6. Place a drop of iodine solution on a slide.
7. Wait 1-2 minutes. Then deftly touch a clean, grease-free coverslip to the meniscus, and gently remove the coverslip with a straight upward motion.
8. If ova or cysts are present they will be contained in the drop which adheres to the underside of the coverslip. Lower the coverslip onto the drop of iodine stain on the slide and examine. Systematically search the entire preparation with the low power objective switching to high dry for observation of details of suspicious objects.

**Zinc Sulfate Levitation (Otto).** This technique is especially useful for the recovery of hook-worm ova in survey work where large numbers of specimens are being examined. Recovery of cysts by this method is also good, but not as satisfactory as zinc sulfate centrifugal flotation techniques.

*Materials and Equipment:*

Applicator sticks  
Vial, approximately 2 inches in height and  $\frac{3}{4}$  inch in diameter  
Zinc sulfate solution, specific gravity 1.18 (75)  
Test tube rack  
Slides  
Coverslips  
Iodine solution (39)

*Technique:*

1. Using 2 applicator sticks, remove a sample of feces about the size of a kidney bean and place it in a vial approximately 2 inches high and  $\frac{3}{4}$  inch in diameter.
2. Fill the tube one-half full with zinc sulfate solution and comminute very thoroughly with the applicator sticks.
3. Place the tube in a test tube rack making sure the tube is perpendicular.

4. Fill the tube brimful with zinc sulfate solution. Carefully apply a coverslip which is larger than the diameter of the tube to the surface of the liquid. There should be no air pockets beneath the coverslip and no overflow of fluid.
5. Allow the preparation to stand for 1 hour. Remove the coverslip with a straight, upward motion and move it to a slide on which a drop of iodine solution has been placed.
6. Examine the preparation under low power covering the entire mount. Cysts and ova should be confirmed under high dry.

**Brine Flotation (Willis-Malloy).** This is a simple, extremely efficient technique for recovery of nonoperculate ova, except for those of the schistosome flukes. It is of no value for recovery of protozoan cysts since they will be unrecognizably shrunken. It would be most valuable, for example, in rapid screening tests for nematode infections in large numbers of individuals.

*Materials and Equipment:*

Applicator sticks

Vial, about 1 inch in diameter, capacity approximately 20 ml

Sodium chloride, saturated solution, specific gravity 1.20

Slides

Coverslips

*Technique:*

1. Using 2 applicator sticks, remove a sample of feces about the size of a marble. Place the specimen in a 1-inch diameter 20 ml vial and fill one-fourth full with salt solution.
2. With the applicator sticks comminute the specimen until the feces is suspended as a fine silt.
3. Fill the vial to the brim with salt solution and then carefully superimpose a glass slide to bring its undersurface into contact with the meniscus. There should be no air pockets beneath the slide and no overflow of fluid.

4. Allow the slide to remain in contact with the meniscus of the fluid for 10 minutes to 1 hour. Optimum time is about 15 minutes.
5. Remove the slide by lifting it straight up without tipping. If ova are present they will be contained in the drop which adheres to the undersurface of the slide. Deftly invert the slide without allowing the drop to run off in the process.
6. Coverslip and examine under low power. Confirm findings under high dry. The preparation should be examined immediately and before the mount commences to dry.

**Acid-Ether Technique (Telemann).** This is a method which is useful for the concentration of all helminth ova except those which bear opercula. It is especially good for the detection of the ova of *Schistosoma mansoni* and *Schistosoma japonicum*. Protozoan cysts are damaged by the acid and this is not a satisfactory method for their recovery. The procedure utilizes an acid to dissolve albuminous matter, soaps, mucin, phosphates and various calcium salts. Ether dissolves the neutral fats and fatty acids. These actions together with mechanical agitation free the ova from the detritus in which they are entrapped. Embryos are killed by the reagent and in schistosomiasis infections, therefore, effectiveness of treatment cannot be determined by this method. It should also be pointed out that the method is expensive when used routinely due to the cost of ether.

*Materials and Equipment:*

Applicator sticks

Test tube, 20 ml

Hydrochloric acid, 15 percent solution

Aqueous Triton N E, 10 percent solution  
(if available)

Gauze

Funnel, 50 mm diameter

Centrifuge tube, graduated, 15 ml

Ether

Stopper for centrifuge tube

Slides

Coverslips

Pipette, capillary with rubber bulb

*Technique:*

1. Using 2 applicator sticks, remove a sample of feces about the size of a marble. If schistosomiasis is suspected, try to secure this entire sample by scraping the outer surface of the stool specimen.
2. Place the fecal specimen in a 20 ml test tube containing 5–10 ml of a 15 percent HCl solution and mix very thoroughly with the applicators. If the reagent is available, it is helpful to add 0.6 ml of Triton N E. This will enhance release of ova from the fecal debris.
3. Strain 5 ml of the fecal suspension through 2 layers of wet cheesecloth or gauze contained in a 50 mm diameter funnel, into a 15 ml graduated centrifuge tube and add an equal quantity of ether.
4. Insert a rubber stopper and shake thoroughly for about 1 minute. Remove the stopper and centrifuge for 1 minute at 1,500 rpm.
5. Four layers should result: a small amount of sediment remaining in the bottom of the tube containing most of the ova, a layer of acid, a plug of detritus just on top of the acid, and a topmost layer of ether.
6. With an applicator stick, ring the plug of debris to free it from the sides of the tube. Quickly and carefully decant the top three layers leaving the sediment undisturbed.
7. Do not rotate the tube back to a vertical position but keep it horizontal. This prevents materials adhering to the side of the tube from flowing back and diluting the bottom sediment.
8. With the tube in horizontal position, insert a long capillary pipette with rubber bulb attached, into the sediment and stir it before withdrawing some into the pipette. Discharge on a clean slide, coverslip, and examine under low power. Confirm findings under high dry.

**Formalin Ether Sedimentation Technique (Ritchie).** In large scale usage this technique has been ascertained to be excellent for the recovery of the cysts of protozoa and it is also very good for the recovery of helminth eggs.

*Materials and Equipment:*

Physiologic saline (54)  
Beaker, 150 ml  
Gauze  
Funnel, 50 mm  
Pointed centrifuge tubes, 15 ml  
Formalin, 10 percent solution  
Ether  
Stopper for centrifuge tube  
Applicator sticks  
Pipette, capillary, with rubber bulb  
Coverslips  
Slides  
Iodine solution (39)

*Technique:*

1. Comminute a portion of stool about the size of a walnut in 10–12 ml of saline in a beaker. Mix thoroughly and pass the mixture through a funnel lined with 2 layers of wet gauze into a 15 ml pointed centrifuge tube.
2. Centrifuge at 1,500–2,000 rpm for 2 minutes. Decant the supernate and resuspend the sediment in fresh saline, centrifuging and decanting as before. This operation is repeated until the supernate is of the desired clarity.
3. Following final decanting add about 10 ml of 10 percent formalin to the sediment, mix thoroughly, and allow to stand for 5 minutes.
4. Add 3 ml of ether, stopper the tube, and shake until thoroughly mixed.
5. Centrifuge at 1,500 rpm for about 2 minutes. Four layers should result: a small amount of sediment containing most of the protozoan cysts and ova, a layer of formalin, a plug of detritus just on top of the formalin, and a topmost layer of ether.
6. With an applicator stick ring the plug of debris to free it from the sides of the tube. Quickly and carefully

decant the top three layers leaving the sediment undisturbed.

7. With an applicator stick mix the sediment remaining in the bottom of the tube with the fluid that drains back from the sides. With a pipette with attached rubber bulb transfer a drop of sediment to a slide, add a drop of iodine and mix thoroughly. Coverslip and examine under low power. Confirm findings under high dry.

**Sedimentation.** This simple technique is applicable to recovery of all ova, cysts and small nematodes. It is a combination of straining and sedimentation which is most useful for recovery of operculate and schistosome ova. Since it is a washing process, concentration, especially in light infections, is not very marked and preparations usually contain much confusing debris. The use of a large fecal sample, however, helps to offset the latter disadvantage.

*Materials and Equipment:*

Tongue depressor  
Beaker, 250 ml  
Glycerin, 0.5 percent solution  
Funnel, 50 mm  
Gauze  
Glass, sedimentation or graduate, cone-shaped  
Pipette, capillary, with rubber bulb attached  
Slides  
Coverslips  
Iodine solution (39)

*Technique:*

1. With a tongue depressor remove a sample of feces about the size of a walnut and place it in a 250 ml beaker. Fill the beaker one-fourth full with a 0.5 percent solution of glycerin in tap water. Tap water may be substituted but glycerinated water tends to reduce surface tension resulting in greater yield of ova.
2. With a tongue depressor mix feces very thoroughly with the diluent,

then add sufficient diluent to fill the beaker three-fourths full.

3. Strain this suspension through a 50 mm glass funnel lined with 2 layers of wet gauze draining the fluid into a conical sedimentation glass or cone-shaped graduate.
4. Allow the suspension to settle for 1 hour and then carefully pour off two-thirds of the supernatant fluid without allowing the sediment to escape.
5. Add fresh diluent and break up the sediment thoroughly to resuspend the sediment uniformly throughout the fluid. Allow settling for an additional hour. The washing procedure should be repeated until the supernatant fluid is relatively clear. However, one washing will generally suffice.
6. Carefully pour off the last wash diluent without losing any of the sediment.
7. Utilizing a capillary pipette with rubber bulb attached remove successively several drops of sediment from the top, center and very bottom of the sediment and transfer to glass slides. Coverslip and examine under low power. Confirm findings under high dry.

*Note:* If a specimen has been previously washed, strained, and fixed in formalin take 10 ml of the thoroughly mixed specimen and start at step 4.

**Merthiolate-Iodine-Formaldehyde-Concentration Technique (MIFC) (Blagg et al.):**

*Materials and Equipment:*

Merthiolate-Iodine-Formalin solution (M.I.F.) (51)  
Applicator sticks  
Gauze  
Centrifuge tube with rubber cork  
Ether  
Slides  
Coverslips

*Technique:*

1. With an applicator stick mix thoroughly 1 part of the fecal suspension with 3 parts M.I.F. solution.

- Preserved in this manner specimens can be stored and examined later.
2. To concentrate the specimen prior to examination, strain this mixture through 2 layers of wet gauze into a 15 ml centrifuge tube.
  3. Add 4 ml of ether to the tube, insert a rubber stopper and shake the tube vigorously. (If ether remains on top after shaking add 1 ml of tap water and reshake.)
  4. Remove stopper and let stand for about 2 minutes.
  5. Centrifuge for 1 minute at 1,600 rpm. Four layers will appear in the tube: an ether layer on top, a plug of fecal detritus, an M.I.F. layer, the sediment containing protozoa and helminth ova on the bottom.
  6. Loosen the fecal plug by ringing with an applicator stick.
  7. Quickly, but carefully, pour off all but the bottom layer of sediment.
  8. Thoroughly mix this sediment, pour a drop on a slide, coverslip and examine.

**Centrifugation.** All helminth ova including those of the schistosomes and cysts can be recovered by this technique. It is thus useful on all specimens regardless of what parasites may be present. Other advantages of this method are that it is rapid, reveals embryos in living condition, does not require expensive reagents to perform, and enhances detection of parasites in low grade infections since a large quantity of feces is used.

*Materials and Equipment:*

Tongue depressor

Test tube, 25 x 150 mm

Stopper, rubber, No. 4

Gauze

Funnel, 50 ml

Tube, centrifuge, 50 ml

Pipette, capillary, with rubber bulb attached

Slides

Coverslips

Iodine solution <sup>3</sup> (39)

<sup>3</sup> Used only on second mount when cysts are observed on initial examination.

*Technique:*

1. With a tongue depressor remove a sample of feces about the size of a walnut. Utilization of a large sample is one of the principal advantages of this technique.
2. Transfer the specimen to a 25 x 150 mm test tube half filled with water and with a tongue depressor mix very thoroughly.
3. Fill the tube two-thirds full with tap water and insert a No. 4 rubber stopper. Shake the tube vigorously for at least 1 minute.
4. Pour the suspension through a 50 mm funnel lined with 2 layers of wet gauze into a 50 ml centrifuge tube. Spin the specimen for 1 minute at a speed of 1,000-1,500 rpm. To avoid collapse of ova, maximum speed indicated here should not be exceeded.
5. Pour off the supernatant fluid. Using a long capillary pipette with rubber bulb attached, take up a few drops of the top of the sediment and transfer it to the center of one end of a slide. Next insert the pipette to the bottom of the sediment removing a few drops. Transfer this sediment to the opposite end of the slide.
6. Coverslip the two preparations, and examine both mounts under low power. Confirm findings under high dry.

*Note:* Make a second mount and stain with iodine if cysts are observed in first preparation. Iodine is omitted in initial examination to avoid killing of miracidia and embryos.

**Centrifugation for Recovery of Ova from Urine**

Ova of *Schistosoma haematobium* are most generally voided in the urine. The last 15-20 ml of urine voided will most often contain the eggs.

*Materials and Equipment:*

Beaker, 50 ml

Tube, centrifuge, 15 ml

Pipette, capillary, with rubber bulb attached  
Slides  
Coverslips

*Technique:*

1. Collect the last 15–20 ml of urine voided into a clean beaker.
2. Transfer urine to a 15 ml centrifuge tube filling it three-fourths full. Centrifuge 1 minute at 1,000–1,500 rpm.
3. Pour off the supernatant fluid. With a capillary pipette fitted with rubber bulb remove several drops of the sediment and transfer to a slide.
4. Coverslip and examine for ova under low power. Confirm findings under high dry.

### **Egg Hatching for Schistosome Miracidia**

In fecal samples containing small numbers of viable eggs of schistosomes, the eggs may be overlooked when routine methods of examination are employed. The procedure here described is especially sensitive and is useful in demonstrating indirectly the presence of schistosome ova when few are present.

*Materials and Equipment:*

Beaker, 250 ml  
Chlorine-free water  
Tongue depressor  
Funnel, 50 mm  
Gauze  
Glass, sedimentation or graduate, cone-shaped  
Flask, Erlenmeyer, 500 ml  
Applicator sticks  
Slides  
Coverslips

*Technique:*

1. Commute a sample of feces about the size of a large walnut in a 250 ml beaker filled one-fourth full with chlorine-free water and mix thoroughly with tongue depressor. The sample should be obtained by scraping the outer surfaces of the fecal mass.

2. Add sufficient water to fill the beaker three-fourths full. Strain this suspension through a funnel lined with 2 layers of wet gauze into a sedimentation glass or cone-shaped graduate and allow the suspension to settle for 1 hour.
3. Carefully pour off the supernatant fluid without allowing the sediment to escape. Add a small quantity of water to the sediment and resuspend it with an applicator stick. Pour the suspension into a 500 ml Erlenmeyer flask and fill it almost to the lip with water. Allow to stand at room temperature overnight.
4. Hatched miracidia will swim about and collect in the neck of the flask thus concentrating them in a small area where they will be more easily visible. Against a dark background the miracidia will be observed as minute, white organisms swimming rapidly in a straight course. Use of a hand lens is helpful.
5. Free living ciliates are easily confused with miracidia. Identification should, therefore, be confirmed by transferring a few drops of fluid containing the organisms to a glass slide. Coverslip and examine. To stop motion of organisms add weak iodine solution or dilute methylene blue. This will enable careful study of the organisms.

### **Techniques for Recovery of Ova of Enterobius Vermicularis**

Ova of pinworms are rarely encountered in routine fecal examinations since the female migrates to the perianal region to deposit ova. Several techniques have been developed for recovery of ova of this species. The basic principle in all of them consists in utilization of Scotch type cellophane tape to which eggs adhere when the sticky side is appressed to the skin in the perianal region.

### **The NIH Technique (National Institutes of Health):**

#### *Materials and Equipment:*

Scotch type cellophane tape

Test tube fitted with a cork stopper through which a small glass rod (approximately 3 inches in length) is inserted

Rubber band

Glass slide

#### *Technique:*

1. Attach a piece of cellophane tape about 1 inch square, sticky side outermost, to the end of a glass rod with a rubber band. The glass rod is passed through a cork which is inserted into a test tube in transit.
2. To obtain the specimen appress the cellophaned tip of the glass rod to several areas in the perianal region.
3. To examine the specimen remove rubber band to free the cellophane paper and spread the paper out smoothly on a glass slide with the sticky side of the paper toward the slide.
4. Examine under low power and confirm findings under high dry.

### **The CDC Technique (Communicable Disease Center):**

#### *Materials and Equipment:*

Scotch type cellophane tape, about three-fourths inch in width

Tongue depressor

Glass slide

Gauze or cotton

#### *Technique:*

1. Place a piece of Scotch type cellophane tape about three-fourths inch in width and about 2 inches in length on a standard sized glass slide, sticky side against the slide, and allow about one-half inch of the tape to overlap the slide at one end.
2. Just prior to taking the specimen hold a tongue depressor flat against the untaped side of the slide with

the tongue depressor protruding about 1 inch below the end where the Scotch tape overlaps. Lift long portion of tape from the slide and loop it over end of depressor to expose gummed surface.

3. Hold tape and slide against tongue depressor with the right hand.
4. Spread the buttocks with the left hand and press gummed surfaces against several areas of the perianal region.
5. Replace tape on slide.
6. Smooth tape with cotton or gauze.
7. Examine under low power and confirm findings under high dry.

*Note* Specimens are best obtained a few hours after the individual has retired or the first thing in the morning before a bowel movement or bath. Details of the proper method of obtaining the specimen are shown in figure 1-1.

### **PVA-Fixative Techniques**

PVA-fixative techniques were developed primarily as a method of preserving trophozoites of intestinal amoebae. Trophozoites disintegrate rapidly after passage from the host and it is, therefore, often difficult to obtain laboratory confirmation of suspected cases of amoebic dysentery or amoebiasis. By utilizing PVA-fixative it is possible to preserve these fragile organisms for subsequent examination. Suspected specimen material can be preserved and either forwarded to a central laboratory for confirmation or stained at a later time and trophozoites if present can be demonstrated. Other uses of this method include the preservation of material for recheck in cases diagnosed as positive as well as the preservation of good specimens for preparation of slides for teaching and reference purposes.

#### **PVA-Fixative Technique (Goldman and Brooke):**

##### *Materials and Equipments*

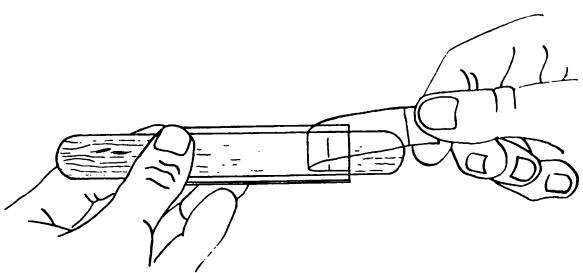
Slides

Coverslips

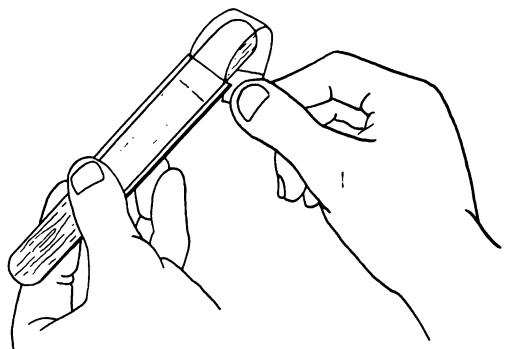
Applicator sticks

Medicine dropper

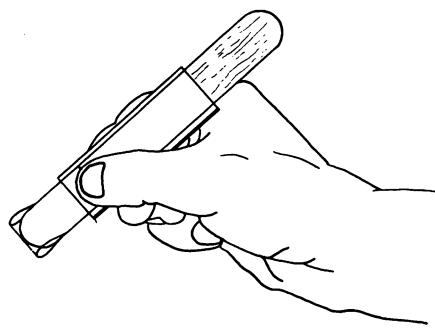
PVA-fixative, prepared solution (56)



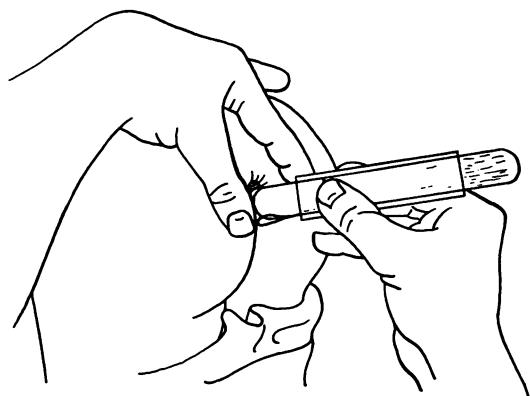
a. Hold slide against tongue depressor and lift long portion of tape from slide.



b. Loop tape over tongue depressor to expose gummed surface.



c. Hold tape against tongue depressor.



d. Touch gummed surface several times to perianal region.

**Figure 1-1. Technique for Recovery of Pinworm Ova.**

*Technique:*

1. It is most important to fix specimens immediately after passage from the patient before the organisms lose their characteristic morphology.
2. Fixation is accomplished by placing a drop of dysenteric stool or a small portion of normal stool on a slide with an applicator stick. With a medicine dropper add 3 drops of fixative.
3. Mix feces and fixative thoroughly with the applicator stick and smear the mixture over an area about an inch square in the center of the slide.
4. Place slides in slide boxes and cover to protect from dust. Allow slides

to dry for several days at room temperature or overnight in a 37° C. incubator. Dried smears remain satisfactory for staining for many months.

5. PVA smears are permeable to all commonly employed staining reagents. They are handled in the same manner as smears fixed with Schaudinn's fixative. Staining utilizing the long Heidenhain iron-hematoxylin procedure gives the best results. Any of hematoxylin staining procedures described later in this section can be followed.

**PVA-Fixative Technique (Ball).** While the original PVA technique described above is ex-

cellent for trophozoites, cysts tend to collapse and also to stain unevenly when that method is followed. The technique described below preserves both cysts and trophozoites.

*Materials and Equipment:*

- PVA-fixative, prepared solution (56)
- Applicator sticks
- Slides
- Centrifuge tubes
- Pipette with rubber bulb attached
- Alcohol, ethyl, 95 percent
- Petri dishes
- Alcohol, 70 percent with 0.5 gm iodine added to each 100 ml alcohol

*Technique:*

1. Place a drop of PVA-alcohol fixative on the center of a slide. With an applicator stick spread the fixative evenly over an area about 22 x 22 mm. Dry the film in a 37° C. incubator overnight.
2. Fix specimen by mixing 3 parts of PVA-fixative solution to 1 part of feces. Specimens fixed in this manner can be either mounted immediately or kept indefinitely.

3. Preparatory to mounting the specimen (a mixture of 1 part feces and 3 parts PVA-fixative) place it in a centrifuge tube and centrifuge at 2,000 rpm for 5 minutes.
4. Decant the supernatant fluid. With a pipette with a rubber bulb attached withdraw a drop of the sediment and place it on the dried PVA-fixative film. With an applicator stick spread the specimen thinly and evenly over the dried film.
5. Place the slide face down in a Petri dish containing 95 percent alcohol for about 10 minutes. Support the slide on broken applicator sticks or toothpicks to prevent the smear from coming into contact with the bottom of the dish.
6. Transfer the slide to 70 percent iodized alcohol for 5 minutes. From this point proceed with the step following 70 percent iodized alcohol in either short or long iron-hematoxylin techniques.

## PREPARATION AND EXAMINATION OF PERMANENT MOUNTS

The most satisfactory and most frequently employed permanent stain for intestinal protozoa is iron-hematoxylin. Various modifications of this staining technique have been developed. Fundamental steps in the various modifications include fixing, mordanting, staining, destaining, and dehydrating. For permanence of stain and definitive morphology the longer methods give the best results. Where permanence of stain is not too important a consideration, more rapid methods can be used to advantage.

Several points regarding the use of these procedures are stressed. The stool specimen should be freshly passed and must not be one obtained following purgation, administration of barium, or oil. The smear should be thin and made on a clean grease-free slide with a toothpick or applicator stick. At no stage in the entire process should the preparation be allowed to dry. Slides which dry at any stage

in the procedure should be discarded. If the decolorizer is not thoroughly washed out of the specimen it will fade the stain. If dehydration is not complete, the xylol will become milky, and, when viewed microscopically after mounting, the preparation will appear blurred.

### Iron-Hematoxylin Stains

**Iron-Hematoxylin—Long Method:**

- Materials and Equipment:*
- Coplin jars or staining dishes
  - Slides
  - Coverslips
  - Applicator sticks
  - Schaudinn's fixative (5 ml acetic acid added to each 100 ml fixative just before use) (63)
  - Alcohol, ethyl, 70 percent, iodized (0.5 gm iodine crystals added to each 100 ml of alcohol)

Alcohol, ethyl, 50 percent  
Iron alum, 4 percent solution  
Hematoxylin, 0.5 percent solution  
Iron alum, 2 percent solution  
Alcohol, ethyl, 70 percent (plus a few drops saturated lithium carbonate)  
Alcohol, ethyl, 95 percent  
Alcohol, ethyl, absolute  
Carbol-xylol (18)  
Xylol  
Balsam, clarite, or other mounting medium dissolved in xylol

*Technique:*

1. Prepare at least 5 smears on each specimen. Streak the feces on the center of the slide with an applicator stick spreading it over an area about the size of a quarter. Streaking should be lengthwise to the long axis of the slide since smears made in this manner are easier on the eyes of the examiner. Proper streaking also produces variations in thickness of the preparations resulting in intergrading differences in staining intensity.
2. Without allowing the specimen to dry place the slides immediately into Schaudinn's solution. The entire procedure can be carried out either in Coplin jars or staining dishes. Fixation can be hastened by heating the fixative to 50° C. and fixing for 5 minutes, or it can be carried out at room temperature for 1 hour.
3. Transfer slides to iodized 70 percent alcohol for 5 minutes. (The iodine combines with mercury to precipitate excess mercury as mercuric iodide.)
4. Transfer the slides to 50 percent alcohol for 3 minutes and then to tap water for 3 minutes.
5. Transfer slides to 4 percent iron alum mordant. Mordanting can be hastened by heating to 40°-50° C. for 10-20 minutes or the process can be carried out at room tem-
- perature by leaving slides in mordant solution overnight.
6. Place slides in 2 successive changes of tap water or distilled water to wash out mordant. Total time for these two changes should be about 3 minutes.
7. Stain slides by immersing for 5-10 minutes in 0.5 percent hematoxylin heated to 40°-50° C. or stain them overnight at room temperature.
8. Place slides in 2 successive changes of tap water or distilled water to wash out excess stain. Total time for these 2 changes should be about 3 minutes.
9. Destain to desired color intensity by transferring slides to 2 percent iron alum. This is one of the most critical steps in this technique. Factors influencing destaining time include the thickness of the smear, the organisms, and the reactions of the particular lots of reagents worked with. The process generally takes from 1-5 minutes. This step should be controlled by timing the destaining on the first slide processed; the destaining is observed microscopically. Time destaining on remaining slides accordingly.
10. Wash destaining solution out thoroughly utilizing several successive changes of distilled or tap water or placing the vessel containing the slides under running tap water. Wash from 5-30 minutes depending upon permanence of stain desired.
11. Transfer slides to 70 percent alcohol-lithium carbonate solution and leave for 3 minutes. Lithium carbonate intensifies the blueness of the stain.
12. Transfer to 95 and 100 percent alcohol for 3 minutes each and then to carbol-xylol for 5 minutes.
13. Clear in xylol for 3 minutes.

14. Mount in balsam, clarite, or other medium dissolved in xylol. About 3 drops of medium are adequate. Excessive amounts make slides difficult to work with due to the fact that excess medium runs out from beneath coverslips. For more rapid drying, slides can be placed horizontally in a 37° C. incubator.

#### **Modified Heidenhain Stain (Diamond):**

##### *Materials and Equipment:*

Coplin jars or staining dishes  
Slides  
Coverslips  
Applicator sticks  
Physiological saline (54)  
Schaudinn's fixative (5 ml acetic acid added to each 100 ml fixative just before use) (63)  
Alcohol, ethyl, 50 percent  
Iron alum, 4 percent solution  
Tergitol-hematoxylin (68)  
Picric acid, saturated aqueous solution  
Alcohol, ethyl, 70 percent with potassium acetate added  
Alcohol, ethyl, 95 percent  
Alcohol, ethyl, absolute  
Xylol  
Balsam, clarite, or other mounting medium dissolved in xylol

##### *Technique:*

1. Emulsify a small bit of feces about the size of a pea in physiological saline. Avoid making emulsion too fluid or too viscous.
2. With a wooden applicator stick, spread a thin even film of the emulsion on a chemically clean glass slide.
3. Fix immediately for 2 minutes in Schaudinn's fixative heated to 60° C.
4. Transfer to 50 percent alcohol for 1 minute and follow with a rinse in distilled water.
5. Mordant for 5 minutes in 4 percent iron alum, followed by a quick rinse in distilled water.

6. Stain for 5 minutes in tergitol-hematoxylin.
7. Rinse in distilled water and destain in a saturated aqueous solution of picric acid. This step will take 3-5 minutes depending upon the thickness of the smear.
8. Rinse in 2 changes of distilled water of about 30 seconds each.
9. Set stain by bluing in 70 percent alcohol to which has been added a few mg of potassium acetate to raise the pH above 7. Bromthymol blue or phenol red may be used as an indicator.
10. Pass successively through 70 percent alcohol, 1 minute; 95 percent alcohol, 1 minute; absolute alcohol, 2 minutes; and xylol, 2 changes of 1 minute each. Mount in any medium dissolved in xylol.

#### **Modified Heidenhain Stain (Markey et al.):**

##### *Materials and Equipment:*

Physiological saline (54)  
Applicator sticks  
Slides  
Coverslips  
Coplin jars or staining dishes  
Schaudinn's fixative (5 ml acetic acid added to each 100 ml just before use) (63)  
Alcohol, ethyl, 95 percent, iodized (0.5 gm iodine crystals added to each 100 ml of alcohol)  
Iron alum, 4 percent solution  
Hematoxylin, 0.5 percent solution  
Alcohol, ethyl, 95 percent  
Alcohol, ethyl, absolute  
Xylol  
Balsalm, clarite, or any other medium dissolved in xylol

##### *Technique:*

1. Emulsify a small portion of feces about the size of a pea in physiological saline. Avoid making emulsion too fluid or too viscous.
2. With a wooden applicator stick, spread a thin even film of the emulsion on a chemically clean glass slide.

3. Fix smears in Schaudinn's for 2 minutes or more.
4. Immerse in 95 percent iodized alcohol for 1 minute.
5. Rinse in tap water.
6. Mordant for 3 minutes at 56° C. in iron alum, and follow with a rinse in tap water.
7. Stain for 2 minutes at 56° C. in 0.5 percent iron hematoxylin.
8. Rinse in water and allow to stand until blue-black. To prevent fading slides should be washed in running water for 15-30 minutes.
9. Dehydrate in 95 percent alcohol for 1 minute, followed by absolute alcohol or acetone for 1 minute.
10. Clear in xylol and mount in any medium dissolved in xylol.

**Modified Heidenhain Stain (Tomkins et al.):**

*Materials and Equipment:*

- Physiological saline (54)
- Applicator sticks
- Slides
- Coverslips
- Coplin jars or staining dishes
- Schaudinn's fixative (5 ml acetic acid added to each 100 ml fixative just before use) (63)
- Alcohol, ethyl, 70 percent
- Alcohol, 70 percent, iodized (0.5 gm iodine crystals added to each 100 ml of alcohol)
- Alcohol, ethyl, 50 percent
- Iron alum, 4 percent solution
- Hematoxylin, 0.5 percent solution
- Phosphotungstic acid, 2 percent solution
- Alcohol, ethyl, 70 percent, plus a few drops of saturated lithium carbonate
- Alcohol, ethyl, 95 percent
- Alcohol, ethyl, absolute
- Carbol-xylol (18)
- Xylol
- Balsam, clarite or other mounting medium dissolved in xylol

*Technique:*

1. Emulsify a small portion of feces about the size of a pea in physiological saline. Avoid making emulsion too fluid or too viscous.

2. With a wooden applicator stick, spread a thin even film of the emulsion on a chemically clean glass slide.
3. Fix smears in Schaudinn's solution for 5 minutes at 50° C. or fix for 1 hour at room temperature.
4. Transfer smears to iodized 70 percent alcohol for 5 minutes.
5. Move smears to 50 percent alcohol for 3 minutes.
6. Rinse smears in tap water.
7. Transfer smears to 4 percent iron alum mordant for 5 minutes.
8. Rinse smears 2 times in tap water.
9. Stain in 0.5 percent hematoxylin for 1 minute and rinse again in tap water.
10. Transfer preparations to 2 percent phosphotungstic acid destaining solution. Leave smears in this solution for a minimum of 2 minutes. With this method destaining is automatic and does not require control by microscopic observation.
11. Rinse smears in running tap water for 5 minutes.
12. Transfer smears to 70 percent alcohol-lithium carbonate solution and leave for 3 minutes.
13. Transfer smears successively to 95 and 100 percent alcohol for 3 minutes each, carbol-xylol for 5 minutes and xylol for 5 minutes.
14. Mount preparations in any medium dissolved in xylol.

**Tannic Acid Fixative Method for Staining Protozoa (Alli):**

*Materials and Equipment:*

- Applicator sticks
- Slides
- Coverslips
- Petri dishes
- Tannic acid fixative (67)
- Iron alum, 4 percent solution
- Hematoxylin, 0.5 percent solution
- Decolorizer (1 percent solution of acetic acid in 70 percent alcohol)
- Alcohol, ethyl, 50 percent

Alcohol, ethyl, 70 percent (with trace of potassium acetate added)

Alcohol, ethyl, 80 percent

Alcohol, ethyl, 95 percent

Alcohol, ethyl, absolute

Carbol-xylol (18)

Balsalm, clarite or any other mounting medium dissolved in xylol

*Technique:*

1. With a wooden applicator stick make 6-12 smears on standard sized round or square coverslips. Handle coverslips with forceps in all steps in this procedure. Drop coverslips face down in a Petri dish one-half filled with tannic acid fixative. (Entire procedure is carried out in Petri dishes.) Let stand 5-7 minutes.
2. Transfer coverslips face down to Petri dish containing tap water. Rinse thoroughly by changing tap water 3 or 4 times.
3. Transfer coverslips to iron alum mordant solution turning them face up as they are transferred. Let stand for 5 minutes. The mordant solution is discarded after each use.
4. Transfer coverslips face up to Petri dish containing tap water. Rinse thoroughly by changing water 3 or 4 times.
5. Transfer coverslips to Heidenhain's hematoxylin face up.
6. Transfer coverslips to Petri dish containing tap water. Rinse thoroughly by changing water 2 or 3 times.
7. Transfer coverslips face up to decoloring solution and allow to decolorize from 30 seconds to 1 minute (never longer than 3 minutes) until desired blue color appears.
8. Pass coverslip face up through 2 changes of 50 percent alcohol, then to 70 percent alcohol with a trace of potassium acetate added (to intensify blueness) and on through 80 percent and 95 per-

cent alcohol. Leave the preparation in each for about 30 seconds.

9. Pass the preparations face up to absolute alcohol and leave for several minutes.
10. Pass through 2 dishes containing carbol-xylol leaving cover slips in each dish about 3 minutes. Mount in any medium dissolved in xylol by turning coverslips face down onto 2 or 3 drops of medium on a slide.

**Concentration and Permanent Staining of PVA-Preserved Fecal Specimens:**

*Materials and Equipment:*

Beaker, 50 ml

Applicator sticks

Funnel, 50 ml

Gauze

Ether

Centrifuge tube, with cork

Cleansing tissue

Slides

Coverslips

Iodine stain or iodized alcohol, 70 percent (see steps 7 and 8 below)

*Technique:*

1. With applicator sticks emulsify 1-2 grams of the preserved fecal specimen in 10 ml of tap water in a 50 ml beaker.

*Note:* Specimens are preserved by mixing 1 part of feces to 3 parts PVA solution.

2. Line a 50 ml funnel with 1 thickness of gauze and filter the preparation.
3. Transfer the filtrate to a centrifuge tube and add 1 ml of ether to the suspension, cork it and shake it vigorously.
4. Centrifuge the preparation for 3-5 minutes at 2,400 rpm. With an applicator stick ring the plug of detritus and discard the supernate and plug retaining only the sediment in the bottom of the centrifuge tube.
5. Transfer the sediment to a piece of cleansing tissue to absorb the excess PVA. Leave the sediment on

- the cleansing tissue for a period of 3-5 minutes.
6. Smear the moist residue on a slide with an applicator stick.
  7. To make a direct examination mix the concentrated fecal specimen with a drop of temporary iodine stain, coverslip and examine.
  8. To prepare a permanent mount place the smear in iodized alcohol, 70 percent and proceed from this step following any of the hematoxylin staining procedures or the trichrome staining procedure described elsewhere in this manual.

**Phosphotungstic Acid Hematoxylin Technique for Staining Intestinal Protozoa:**

*Materials and Equipment:*

Applicator sticks  
Slides  
Coverslips  
Coplin jars  
Schaudinn's fixative (63)  
Alcohol, ethyl, 30, 50, and 70 percent  
Alcohol, ethyl, absolute  
Alcohol, ethyl, 70 percent, iodized  
Sodium hyposulfite solution (66)  
Hematoxylin stain (36)  
Xylool  
Balsam, synthetic resin or other suitable medium

*Technique:*

1. Emulsify a bit of feces about the size of a pea in physiological saline. Avoid making the emulsion too fluid or too viscous.
2. With an applicator stick spread a thin film of the emulsion on a chemically clean glass slide.
3. Fix in Schaudinn's fixative for 30 minutes.
4. Pass slides to 70 percent ethyl alcohol for 10 minutes.
5. Transfer slides to sodium hyposulfite solution for 10 minutes.
6. Remove sodium hyposulfite by immersing slides in two changes of 70 percent ethyl alcohol, 2 minutes each (manually agitate slides in

this solution occasionally during this time interval).

7. Immerse slides in absolute ethyl alcohol for 10 minutes to harden parasites.
8. Transfer slides consecutively to 70, 50, and 30 percent ethyl alcohol, 3 minutes each.
9. Place in hematoxylin stain for 12 hours or overnight at room temperature or in a 37° C. incubator for 4 hours.
10. Wash in tap water for 1 minute.
11. Dehydrate rapidly by successive transfer of slides to 30, 50, and 70 percent alcohol for 1 minute each.
12. Pass slides through 2 successive changes of absolute ethyl alcohol, 3 minutes each.
13. Clear in 2 successive changes of xylool for 5 minutes each.
14. Cover mount with 2 or 3 drops of synthetic resin or Canada balsam, and coverslip preparations.

**Acid Fuchsin Fast Green Staining of Intestinal Protozoa (Lawless).**

*Materials and Equipment:*

Applicator sticks  
Slides  
Coverslips  
Medicine dropper  
Acid Fuchsin-fast green fixative (2)  
Alcohol, ethyl, 50, 70, 95 percent  
Alcohol, ethyl, absolute  
Xylool  
Balsam, damar, or other suitable medium dissolved in xylool

*Technique:*

1. Using an applicator stick, smear a thin layer of feces on the slide. At no time in the procedure should the smear be allowed to dry.
2. Using a medicine dropper, immediately cover the preparation with the fixative-stain, making sure the preparation is completely covered.
3. Gently heat the stain covered smear over a burner or alcohol lamp until steam is first observed. Two or three passes of the flame are usually

sufficient. *Do not boil or allow the solution to flame.*

4. Wash immediately in tap water.
5. Place in 50 and 70 percent alcohol for 30 seconds each.
6. Transfer slides to 95 percent and absolute alcohol for 15 seconds each.
7. Clear the preparations in xylol for 1 minute or more.
8. Mount in balsam, damar, or other suitable medium dissolved in xylol.

**Preparation of Smears from Zinc Sulfate Concentrated Stools for Trichrome or Hematoxylin Staining:**

*Materials and Equipment:*

Wire loop  
Human serum  
Slides  
Coverslips  
Schaudinn's fixative solution (63)

*Technique:*

1. Prepare zinc sulfate concentrated stool according to method described elsewhere in this manual.
2. Allow several minutes after concentration of the stool to permit time for parasites to float to the surface.
3. With a wire loop rapidly apply a loopful of human serum to the surface film of the concentrated stool.
4. After allowing time for the serum to spread over the meniscus touch a wire loop approximately 5 mm in diameter to the surface film removing several loopfuls in this manner to a glass slide. It is important that the loop be parallel to the surface film and the holder held perpendicular to the film.
5. The serum and adherent material is smeared with a rotational movement over the glass slide.
6. Fix preparations in Schaudinn's fixative for 30 minutes.
7. Stain with either hematoxylin or trichrome stain following any of the methods described in this manual. (Proceed with step following fixation.)

*Note:* Formalized, ether extracted, and PVA-preserved stools do not lend themselves to this method.

**The Preparation and Trichrome Staining of PVA-Preserved Specimens or Schaudinn's Fixed Fresh Stools:**

*Materials and Equipment:*

Slides  
Coverslips  
Alcohol, ethyl, iodized, 70 percent  
Alcohol, ethyl, 70 percent  
Trichrome stain (69)  
Alcohol, ethyl, 90 percent, acidified  
(Acidify by adding 1 drop of glacial acetic acid to each 10 ml of alcohol)  
Alcohol, ethyl, absolute  
Xylol  
Xylo-damar, balsam, permount or other suitable mounting medium

*Technique:*

1. Place PVA or Schaudinn's fixed preparations in 70 percent iodized ethyl alcohol for 1 minute. (See technique for preparation of PVA smears described elsewhere in this manual.)
2. Pass specimens to 70 percent ethyl alcohol for 1 minute.
3. Pass specimens through a second change of 70 percent ethyl alcohol for 1 minute.
4. Place specimens in trichrome stain for 5-10 minutes. The shorter period of time is satisfactory if stools are very soft or liquid.
5. Pass preparations to 90 percent acidified ethyl alcohol for 10-20 seconds or until the stain barely runs from the smear.
6. Rinse slides in 2 changes of absolute ethyl alcohol.
7. Pass slides to fresh absolute ethyl alcohol and leave for 1 minute.
8. Clear preparations in xylol for 1 minute or longer. The slides should not be removed from the xylol until the preparations are clear.
9. Add 2 or 3 drops of permount or other suitable mounting medium and coverslip.

## THE CULTURE OF INTESTINAL PROTOZOANS

A number of different media have been developed for the culture of intestinal protozoa. Claims to the effect that one or the other of these media is superior to the other are probably unfounded. Different degrees of success with the different types probably represent differences in the amount of experience acquired in working with them. Their value as diagnostic procedures has been questioned by many workers. There are some who point out that culture techniques are most time-consuming and that an equivalent amount of time spent in examination of a larger number of stools on each patient and a more thorough search of each specimen will serve to detect a larger number of positives. A technician's actual experience in routine follow-up of specimens found negative by other techniques will serve to indicate whether routine culture of such specimens is worthwhile. Three of the more commonly used media will be outlined here.

For the benefit of persons who have had no prior experience with protozoan culture several points are stressed. With the exception of *Giardia lamblia* and *Balantidium coli* all human intestinal protozoa can be cultured. There are differences in culturability of different strains of protozoa within the same species. Large strains of *Endamoeba histolytica*, for example, are more readily cultured than the smaller strains. Some strains cannot be cultured at all while others are very readily cultured. Trophozoites, especially those of amoebae, tend to be atypical in culture, presenting nuclear morphology quite different from that seen in trophozoites in stools. Different protozoa are more readily recovered from different levels in the media. Amoebae in general will be found at the very bottom of the fluid, *Chilomastix mesnili* is found most abundantly at the middle level, while *Trichomonas hominis* occurs in greatest abundance near the upper surface. Negative findings in cultures cannot be accepted as an indication that a patient is free from infection.

In examining protozoan cultures for organisms it is most important that the mounted

preparation be kept warm during examination. The slide should be warmed in the incubator to 37° C. before the specimen is transferred thereto. The examination should be performed in a warm room. After the mount is prepared it should be returned to the incubator for about 5 minutes to allow time for the organisms to commence normal locomotion.

Protozoan cultures are often maintained in laboratories for teaching purposes. Such cultures must be transplanted three times weekly and are generally serially transferred on Mondays, Wednesdays, and Fridays. The quantity of inoculum utilized is about 0.5 ml, and location in the culture from which the inoculum is taken must take into account the growth habits of the particular species involved. To assure against loss of cultures, the old cultures are retained until a subsequent transfer is made from the new ones. The cultures should be checked each time prior to transfer. Maintenance of such cultures in the laboratory is not easy and offers a real challenge to the technician.

Principal sources of difficulty in the culture of protozoans are:

1. *In the preparation of the medium:*
  - a. Fresh eggs (not more than 1 day old) should be used. A "blood spot" in a fresh egg is not harmful.
  - b. Chemically clean glassware should be used exclusively. Wherever possible all glassware and equipment used in preparation of medium should be sterile. This will prevent excessive contamination which often cannot be effectively eliminated by the maximal sterilization which the medium will tolerate.
  - c. If an autoclave must be used for inspissating medium, temperature and pressure must be most carefully controlled or the medium may bubble, be scorched, or inadequately sterilized. Commercial inspissators are much more satisfactory if available.
  - d. The pH is critical and must be maintained at the specified level.

- e. Medium should be checked for sterility, refrigerated, and used within three weeks after preparation.
2. *In inoculation and transfer:*
  - a. Wherever possible inoculation and transfer of cultures should be performed in an area where there is the least chance of aerial contamination.
  - b. Cultures should not be allowed to stand at room temperature any longer than necessary.
  - c. In transferring cultures the inoculum must be taken from the proper depth.
  - d. When cultures are being maintained the time interval between transfers should not exceed 72 hours.
3. *In identifying the species from culture material:*
  - a. Trophic forms are frequently so atypical both in morphology and motility that they cannot be differentiated on direct examination.
  - b. Cultures in which trophic forms are found should be incubated for an additional 24 hours and examined for cysts. The latter display characteristic morphology and permit accurate identification.

## **Media for Culture of Protozoans**

**Boeck and Drbohlav's Medium. Modified Locke's Solution for Boeck and Drbohlav's Medium:**

### *Materials and Equipment:*

Distilled water.....	1,000.00 ml
Sodium chloride.....	8.00 gm
Calcium chloride.....	0.20 gm
Potassium chloride.....	2.20 gm
Magnesium chloride.....	0.01 gm
Disodium hydrogen phosphate .....	2.00 gm
Sodium bicarbonate.....	0.40 gm
Potassium dihydrogen phosphate .....	0.30 gm
Flask, Erlenmeyer.....	2000 ml
Funnel .....	150 ml
Filter paper	

### *Technique:*

1. Add chemicals to the distilled water in the order listed. After adding each chemical mix thoroughly until dissolved. Heat for 20 minutes.
2. A precipitate will form. Cool to room temperature and filter through paper.
3. Dispense in suitable sized containers and sterilize in autoclave for 15 minutes at 15 pounds of pressure. Final pH should be 7.5.

### *Preparation of Slants:*

#### *Materials and Equipment:*

Fresh eggs.....	4
Sterile Locke's solution . . .	50 ml
Alcohol, ethyl.....	70 percent
Flask .....	250 ml
Glass beads	
Funnel .....	50 ml
Gauze	
Test tubes.....	15 ml
Cotton, absorbent	

### *Technique:*

1. Wash 4 fresh eggs with soap and water, rinse in 70 percent alcohol.
2. Break the eggs into a flask with beads. (A Waring blender should be used if available.)
3. Add the sterile Locke's solution and emulsify.
4. Filter the mixture through gauze and dispense 5 ml quantities of the medium into 15 ml test tubes and plug the tubes with absorbent cotton.
5. Inspissate in the autoclave.
6. Place the tubes in the chamber in the desired slanted position. If the autoclave has a jacket, allow the pressure to reach 15 pounds. Close all exhaust valves and the door of the chamber. This procedure will entrap all the air in the chamber. Allow the steam from the jacket to enter the chamber as fast as possible until pressure gauge registers 15 pounds. Hold at this pressure for 15 minutes. Cut off steam supply and allow pressure to drop

gradually with all doors and valves tightly closed.

7. If the autoclave has no jacket, place the tubes in the chamber, close all exhaust valves and the door tightly. (If the drain at the bottom of the autoclave has no valve, it may be stoppered with a rubber stopper.) Turn on the steam and allow the pressure to rise to 15 pounds. (The temperature of the mixture of air and steam will not be above 85° C.) Hold at this pressure for 15 minutes. Turn off steam and allow autoclave to cool gradually. Sudden drops of pressure tend to disrupt the medium causing bubbles and/or rough surfaces.
8. When the inspissated slants are cold, cover them to a depth of 1 cm with sterile Locke's solution. Sterilize in the autoclave at 15 pounds pressure for 15 minutes. Allow pressure to drop very gradually. Test for sterility by incubating at 37° C. for 24 hours, and store tubes in refrigerator until needed.

*Note:* Just prior to use add 2 or 3 loopfuls of sterile rice flour to fluid overlay in each tube.

#### **Modified Cleveland and Sanders Medium for Endamoeba Histolytica.**

##### *Serum-saline Solution:*

###### *Materials and Equipment:*

Na <sub>2</sub> HPO <sub>4</sub> .12H <sub>2</sub> O.....	11.23 gm
KH <sub>2</sub> PO <sub>4</sub> .....	0.26 gm
NaCl.....	8.00 gm
Distilled water.....	1,000.00 ml
Sterile horse serum.....	100.00 ml
Flask, Erlenmeyer .....	2000.00 ml

###### *Technique:*

1. Dissolve the three salts in the distilled water and autoclave this solution at 15 pounds pressure for 20 minutes.
2. Cool and add the sterile horse serum. Add sufficient serum-saline solution to each slant to cover about three-fourths of the slant.

#### *Base Medium:*

##### *Materials and Equipment:*

Entamoeba medium, dehydrated (Difco).....	33 gm
Distilled water.....	1,000 ml
Flask, Erlenmeyer .....	2000 ml
Test tubes .....	15 ml
Cotton, absorbent	

###### *Technique:*

1. Dissolve the dehydrated medium in the distilled water, dispense the medium in 5 ml quantities in 15 ml test tubes, plug with absorbent cotton, and autoclave at 15 pounds pressure for 20 minutes.
2. Slant and leave at room temperature for several days until the slants are hardened.
3. Incubate overnight at 37° C. to test for sterility. Store in refrigerator.

*Note:* Just prior to use add 2 or 3 loopfuls of sterile rice flour to fluid overlay in each tube.

#### **BALAMUTH'S EGG INFUSION MEDIUM.**

##### *Materials and Equipment:*

NaCl solution 0.8 percent.	125 ml
Wilson's dry extract of liver (powder) .....	5 gm
Distilled water.....	120 ml
Boiled yolks of fresh eggs (boil 15 minutes) .....	4
M/15 Potassium phosphate solution buffered to pH 7.5	
Waring blender	
Double boiler with cover	
Buchner funnel	
Filter paper.....	#2
Test tubes .....	15 ml
Cotton, absorbent	

###### *Technique:*

1. Mix the egg yolks with the 0.8 percent NaCl in a Waring blender.
2. Heat the mixture in a covered double boiler. When the temperature reaches 80° C. add 20 ml of the distilled water to make up for evaporation.
3. Filter the mixture through a Buchner funnel under reduced pressure using several layers of #2 filter

paper. Autoclave the filtrate for 20 minutes at 15 pounds pressure. Cool to below 10° C. and refilter through Buchner funnel. Measure the filtrate and add an equal quantity of M/15 potassium phosphate solution.

4. To 100 ml of distilled water add 5 gm of dry extract of liver and bring it to a boil.
5. Filter and autoclave the filtrate at 15 pounds pressure for 20 minutes.
6. The two solutions are now mixed in the ratio of 1 part of liver extract solution to 9 parts of the egg yolk infusion.
7. Autoclave again at 15 pounds pressure for 20 minutes.
8. Tube in sterile test tubes in 5 ml amounts, plug the tubes with absorbent cotton, incubate medium for 24 hours at 37° C., and store in the refrigerator.

*Note:* Two or 3 loopfuls of sterile rice starch are added to the medium just before use.

#### **Inoculation and Examination of Cultures for Intestinal Protozoans:**

##### *Materials and Equipment:*

Boeck and Drbohlav's, Balamuth's, or Cleveland and Sanders' medium  
Sterile rice powder  
Platinum loop  
Penicillin and Streptomycin solution (5000 units of each per ml)  
Pipette, large bore, with attached rubber bulb  
Applicator sticks

##### *Technique:*

1. Prior to inoculation tube medium is placed in a 37° C. incubator for a few hours to bring the medium up to incubating temperature.
2. Add 2 or 3 loopfuls of sterile rice flour to each tube of medium just prior to use. (Rice flour is sterilized by dispensing it in approxi-

mately 1 gm quantities in Wassermann tubes and heating in a dry oven for 2½ hours at 150° C. The flour can be conveniently dispensed with a sterile platinum loop. Use a fresh tube of rice flour each time a batch of tubes is inoculated.)

3. Inoculate 2 tubes from each specimen. One of the tubes is treated with antibiotics at the rate of 250 units each of penicillin and streptomycin per ml of fluid in the tube. (5,000 units of each per 1 ml. Therefore, 250 units of each will be contained in .05 ml or approximately 1 drop.)
4. If the stool is fluid, transfer 0.5 ml of the specimen to each tube with a pipette with attached rubber bulb.
5. To inoculate formed stools select a portion of stool about the size of a pea, taking material from several parts of the specimen with an applicator stick. Transfer the feces to the fluid medium and macerate it against the side of the tube to mix it thoroughly.
6. Incubate tubes at 37° C. After 24 hours of incubation check for growth by removing a few drops from the bottom of the tube if utilizing a fluid medium, or from the base of the slant if utilizing a fluid overlay over a hard base medium.
7. If negative recheck the specimen after 48 hours of incubation. Specimens negative after 48 hours can either be discarded or retained for an additional 24 hours and examined once more. Retention of specimens for 72 hours enhance cyst formation.
8. For definitive diagnosis positive specimens can be processed by either of the PVA-fixative techniques discussed elsewhere in this manual.

## **RECOMMENDED ROUTINE FOR STOOL SPECIMENS**

1. Careful and thorough macroscopic examination.
2. Three direct wet smears with saline and iodine-stained halves. (D'Antoni's Iodine solution recommended.)
3. A zinc sulfate flotation preparation. (Faust's method recommended.)
4. Permanent iron-hematoxylin stained slide on all positive specimens for definitive species diagnosis (protozoan cysts and trophozoites) and permanent file record.
5. Acid-ether preparation on cases suspected of harboring operculate or Schistosome ova but not confirmed by routine wet smears. (Telemann's method recommended.)

## Chapter 2

# THE PREPARATION OF PERMANENT MOUNTS OF HELMINTHS

The preparation of permanent mounts of helminths is a laboratory procedure which is quite frequently performed, particularly in large overseas hospitals where helminth infections are frequently encountered. The two principal reasons for making such preparations

are for the laboratory diagnosis of helminth infections based on observation of the morphology of adults recovered in stool examinations, and secondly for the preparation of materials for reference as well as for use in on-the-job training of laboratory personnel.

### PREPARATION OF HELMINTHS FOR STAINING

#### Removal of Helminths From the Host

For preparation of good whole mounts the specimens should, whenever possible, be obtained in living condition. The most suitable material for good mounts is obtained by dissecting freshly killed host animals. Since a large majority of helminths will be found in the intestines, the proper method for removing worms from the alimentary tract will be briefly described.

First, expose the entire viscera. If the host animal is relatively small, tie the gut off with string at the esophageal and rectal ends. Grasp the rectal end with a forceps and with sharp shears carefully cut away the connecting mesenteries and other tissues to free the gut throughout its length. For larger animals tie off and cut out shorter portions thereof and transfer to a dish of physiological saline and with a fine shears start at one end and slit the intestine open lengthwise to expose the lumen. With a forceps agitate the gut gently in the fluid. This will dislodge the free and less securely attached organisms. Forms securely attached by suckers or hooks should not be forcibly dislodged. The latter may be caused to free themselves from attachment by placing the material in the refrigerator for a few hours. Dissecting away the tissue around points of attachment and occasional agitation of the dish will hasten the process. When dealing with

smaller species, the entire contents of the dish should be carefully examined under a dissecting microscope.

To prevent rupture and distortion of freed organisms, transfer them with blunt instruments. Very small organisms can be aspirated and transferred to fresh physiological saline with a wide mouth pipette. If large enough, the worms may be transferred with camel's-hair brush, small spatula, or wire loop. Glass rods or any suitable blunt instrument can be used to handle the larger helminths since they are less fragile.

Helmint inhabiting tissues, such as liver flukes, lung flukes, and cysticerci must be very carefully dissected out. Liver flukes, for example, are the same coloration as the tissue in which they occur and must be carefully searched for. The entire organ is first cut into pieces of approximately one pound each. Each piece is dissected by tearing along the course of the larger ducts, and the worms are squeezed out.

#### Cleansing of Helminths

Following removal from the host, the worms should be transferred carefully through several changes of saline and gently agitated in each change to remove all adhering mucus and debris. A soft brush can be used to remove particles that cannot be loosened by shaking

the dish, the latter being best accomplished under a binocular dissecting scope. If specimens are plentiful, the damaged ones should be discarded at this point.

### **Relaxation of Living Helminths**

Various methods may be used to cause helminths to relax to preserve normal morphology. The technique of choice depends chiefly upon the size and the particular parasite with which one may be dealing.

Very small trematodes can be relaxed by placing them in several drops of saline on a glass slide, coverslipping, exerting gentle pressure on the coverslip, and then cooling in the refrigerator for about an hour. Larger trematodes may be placed in a wide mouth jar half filled with saline and the contents shaken vigorously for several minutes to cause the worms to relax. Large trematodes may also be relaxed by placing them individually or in groups, depending upon size, on large glass slides and a few drops of fixative are then dropped on them. They are then quickly covered with a second glass slide and pressure is immediately applied to flatten them out to normal contour. Tie the slides securely together with string and submerge the entire preparation in the fixative. To prevent distortion, do not tie the slides together too tightly. Separate the slides slightly from time to time and force fixative between them to make certain that all parts of the organism are in contact with the fixative. Cestodes and trematodes may also be relaxed by placing them in physiological saline and leaving them for several hours or overnight in the refrigerator. Some trematodes, particularly schistosomes, tend to deteriorate if retained as long as 10 or 12 hours prior to fixation and these should, therefore, be relaxed for periods not exceeding about 3 hours prior to fixation. The larger tapeworms may be extended to normal length by wrapping them about strips of plate glass or around a large test tube or glass jar. Larger cestodes, the mounts of which will be individual proglottids or short chains of proglottids, can be relaxed by cutting in strips and compressing between two glass slides in the manner previously described for large trematodes. To prevent distortion or loss of

the heads of tapeworms, they should be cut off first and handled individually throughout all procedures preparatory to mounting. Long strips of tapeworm proglottids can be cut from the strobila and placed alongside one another on pieces of heavy paper toweling which has been soaked in fixative. Gently flatten the strips and extend them to normal length. After the worms have been properly extended a piece of fixative-soaked paper toweling can be placed on top of them. After about 30 minutes sufficient additional fixative to cover, but not float, the paper should be added and fixation continued overnight.

Nematodes require no special handling to assure fully extended and relaxed specimens. Following cleansing, one may proceed to fixation without any intermediate steps.

### **Fixation**

The proper fixation of helminths is absolutely essential for permanent preservation of the specimens. Fixation stops metabolic processes quickly and preserves actual form if accomplished when the organisms have been properly relaxed. It hardens the tissues, prevents regressive changes from taking place, and preserves the cytological and histological elements in their natural state. Numerous fixation techniques can be used. The simpler and more common ones will be described here.

Formalin fixation is rapid, simple, and inexpensive. A 5 percent steaming (not boiling) solution of formalin is recommended. Most of the saline is first poured off and the formalin solution is rapidly poured over the extended worms. After several hours, pour off the formalin and add fresh unheated 5 percent formalin. Since formalin is an excellent preservative as well as a fixing agent, the organisms can be left in it indefinitely provided the container is sealed to prevent evaporation. Prior to proceeding with any permanent mounting technique, the formalin must be washed out of the specimens by running tap water over them for several hours. This is easily accomplished by removing the top of the container and substituting several layers of cheesecloth secured in position by string or rubber band. The container is then placed

under a cold water tap and the specimens are washed with slowly running water for several hours.

Other commonly used fixatives which will give more delicate fixation include alcohol-formalin-acetic acid fixative (A.F.A.), Gilson's mixture, and Schaudinn's fluid. The latter two fixatives contain mercury, and specimens must not be handled in them with metal instruments. Only glass rods or wooden sticks should be brought into contact with these fixatives. A.F.A. fixative should be used cold while Gilson's and Schaudinn's may be used either cold or as steaming solutions. If hot solutions are used, the organisms are obtained in a more natural and extended position. At some stage in the processing prior to mounting, specimens fixed in solutions containing mercury must be treated with iodized alcohol to precipitate the mercury. If A.F.A. fixative is used, allow the specimens to remain in the solution overnight and then transfer them directly to 70 percent

alcohol in which they can be left indefinitely. Following fixation with Gilson's or Schaudinn's, transfer the specimens directly to 70 percent iodized alcohol and allow them to remain there with occasional agitation for 12-24 hours depending on the thickness of the organisms. The specimens are next transferred to 70 percent alcohol where they can be left indefinitely. It is stressed at this point that regardless of what fixative is used, specimens will make better mounts if they are processed through to a point where they can be stored in 70 percent alcohol following fixation.

The cuticula of nematodes is quite impervious, and the routine fixation methods discussed above are, therefore, not recommended for fixation. One commonly used technique is to place the living specimens in a small beaker and pour a considerable volume of hot glycerin-alcohol over them. They can be left in the glycerin-alcohol fixative indefinitely provided the container is sealed to prevent evaporation.

## STAINING AND MOUNTING OF HELMINTHS AND HELMINTH OVA

Staining and mounting techniques will vary depending upon size of specimens, species, and the stage of development of the organisms. Various techniques applicable to processing of material, taking these factors into consideration, are set forth in this part. All procedures are written on the assumption that specimens were treated with iodine (if fixed in solutions containing mercury) to precipitate the mercury and then stored in 70 percent alcohol.

### Staining and Mounting of Adult Trematodes

#### Large and Medium-Sized Specimens:

##### Materials and Equipment:

Alcohol, ethyl, 30, 35, 50, 70, 85 and 90 percent  
Distilled water  
Staining dishes  
Stain (any stain listed in step 2)  
Alcohol, ethyl, acidified (add 6 drops of conc. HCl to 100 ml of 35 percent alcohol)

Ammonia water, dilute (add 1 drop of conc. ammonia to 250 ml of distilled water)

Beechwood creosote or aniline oil

Xylol

Xylo-damar or balsam (73)

Slides

Coverslips

Applicator stick with spatulate end

Pipette, wide-mouthed, with attached rubber bulb

#### Technique:

1. Pass the specimens successively through 50 percent and 30 percent alcohol, 1 hour each and wash thoroughly in distilled water.
2. Stain overnight. (Use Mayer's paracarmine, Grenacher's borax carmine, Delafield's hematoxylin Ehrlich's acid hematoxylin, or Roudabush's hematin.)
3. Pass through three successive changes of tap water, 5 minutes each.

4. Destain in acidified 35 percent alcohol. With the carmine stains, destain until the specimens display a delicate pink color. With the hematoxylin stains, destain until the specimens are a light reddish-purple. This is a critical step and should be done under observation.
5. Transfer specimens to ammonia water for 30 minutes.
6. Pass through three successive changes of tap water, 5 minutes each.
7. Dehydrate by passing successively through 35, 50, 70, 85, and 90 percent ethyl alcohol, 30 minutes each.
8. Clear in beechwood creosote or aniline oil for 1 hour.
9. Wash in xylol, two changes, 5 minutes each.
10. Mount in xylo-damar or balsam. The mount is prepared by first transferring a few drops of fairly thick mounting medium to the center of a clean, grease-free slide. Transfer large specimens to the mounting medium on the slide with an applicator stick, the end of which has been cut to form a flat spatulate surface. Smaller specimens can be handled with a wide-mouthed pipette with attached rubber bulb. In transferring specimens from clearing agent to mounting medium, transfer as little xylol as possible to the medium. With the applicator stick, gently press the specimen down into the mounting medium. Care must be taken not to allow specimens to dry at any stage in the process. Additional medium is then added, the amount depending upon size and thickness of the preparation. Cover by bringing one edge of the coverslip into contact with the medium and drop it gradually with a rolling motion to prevent entrapment of bubbles. In mounting large specimens, bits of broken glass can be placed under the margins of the coverslip to prevent it from tipping. Dry in flat position for several weeks. Drying in a 37° C. incubator hastens the process.
11. If specimens turn opaque or white when they are placed in the medium, this is an indication that dehydration was incomplete. Such specimens can be returned to xylol until they are clear and then remounted. Specimens persistently opaquing can usually be cleared by placing them in xylol containing mounting medium. The quantity of medium in xylol is increased gradually. The container is left unstoppered to allow the xylol to evaporate. Specimens mounted in balsam have less tendency to become opaque than specimens mounted in damar.

**Very Small Species of Adult and Larval Trematodes:**

*Materials and Equipment:*

Alcohol, ethyl, 70, 80, 90 percent and absolute

Staining dishes, watch glasses, or evaporating dishes

Pipette with attached rubber bulb

Mayer's paracarmine stain (50)

Alcohol, ethyl, acidified (add 1 ml of conc. HCl to 100 ml of 70 percent alcohol)

Slides

Coverslips

Xylo-damar or balsam (73)

*Technique:*

1. Transfer specimens from the 70 percent alcohol preservative to a small dish or watch glass. The material is very conveniently processed in small round-bottomed porcelain evaporating dishes.
2. Allow time for specimens to settle to the bottom of the container, then carefully pipette off the alcohol leaving only a sufficient

- amount in the bottom of the dish to prevent specimens from drying.
3. If working with very small specimens, check under a binocular dissecting scope to ascertain their presence before proceeding further.
  4. Barely cover the specimens with Mayer's paracarmine and stain for a minimum of 1 hour.
  5. To dilute the stain and minimize danger of loss of small specimens, fill the container with 70 percent alcohol. Allow time for the specimens to settle to the bottom and then pipette off excess alcohol. Fill the container again with 70 percent alcohol and withdraw alcohol by pipetting, repeating until the fluid is colorless. Retain and examine alcohol removed to make certain that no specimens are discarded.
  6. Cover specimens with acidified 70 percent alcohol and destain under observation until specimens are a delicate pink color. Small specimens destain rapidly and must be observed closely to prevent complete bleaching of the stain. Generally, cestodes and small trematodes destain quite rapidly, usually requiring 10–30 minutes, depending on the size. The larger trematodes may take up to several hours to destain.
  7. Pipette off as much of the acid alcohol as possible and cover specimens with 80 percent alcohol. Again remove as much of the alcohol as possible and cover with a fresh change of 80 percent alcohol leaving in this solution for a minimum of 30 minutes. If the alcohol assumes a pinkish hue, this step should be repeated to remove any traces of acid which may be present.
  8. Pass successively through two changes of 95 percent alcohol, leaving in each change for 5 minutes or more.
  9. Transfer specimens to absolute alcohol, leaving 5 minutes or more.
  10. Pass specimens to second change of absolute alcohol, vaseline-seal the container, and let stand overnight. (*Note:* All changes are made by pipetting off as much fluid as possible.)
  11. Remove excess alcohol and pass specimens through two successive changes of xylol of 5 minutes each.
  12. Mount in thin xylo-damar or balsam following method previously described for adult trematodes.

### **Staining and Mounting of Cestodes**

#### **Delafield's Iron-Hematoxylin Stain:**

##### *Materials and Equipment:*

Alcohol, ethyl, 30, 50, 70, 80, and 95 percent

Delafield's hematoxylin stain (21)

Distilled water

Staining dishes

Alcohol, ethyl, acidified (add 1 ml of conc. HCl to 100 ml of 70 percent alcohol)

Alcohol, ethyl, ammoniated (add 1 drop of conc. ammonia to 250 ml of 70 percent alcohol)

Beechwood creosote or aniline oil

Xylol

Xylo-damar or balsam (73)

Slides

Coverslips

##### *Technique:*

1. Transfer specimens (stored in 70 percent alcohol) successively through 50 and 30 percent alcohol for 1 hour each.
2. Dilute stock Delafield's iron-hematoxylin 1–9 with distilled water just prior to use. Transfer specimens to staining solution and stain for several hours or overnight.
3. Wash in several changes of water to remove adhering stain.
4. Pass through successive changes of 30, 50, and 70 percent alcohol for

- a minimum of 30 minutes each. Larger specimens should be left longer in each successive change.
5. Destain in 70 percent acidified alcohol until the specimens assume a delicate light reddish-purple color. If the alcohol becomes deeply colored, it should be changed.
  6. Wash for a few minutes in 70 percent alcohol. Remove specimens to fresh 70 percent alcohol and leave for several hours. If alcohol takes on any coloration, this step should be repeated.
  7. Place specimens in ammoniated 70 percent alcohol until they become bluish-purple.
  8. Dehydrate successively in 80 and 95 percent alcohol for a minimum of 30 minutes each.
  9. Clear in beechwood creosote or aniline oil for 1 hour. Wash in xylool, two changes 10 minutes each.
  10. Mount in xylo-damar or balsam following method previously described for adult trematodes.

#### Semichon's Carmine Stain:

##### *Materials and Equipment:*

Semichon's carmine, stock solution (64)  
Alcohol, ethyl, 70, 80 and 95 percent  
Staining dishes  
Alcohol, ethyl, acidified (add 1 ml conc.  
HCl to 100 ml of 70 percent alcohol)  
Carbol-xylool, beechwood creosote, or aniline oil  
Slides  
Coverslips  
Xylo-damar or balsam

##### *Technique:*

1. Mix Semichon's carmine stock solution in equal parts with 70 percent alcohol. Transfer specimens directly from 70 percent alcohol preservative to staining solution and allow to stain overnight.
2. Wash in several changes of 70 percent alcohol, 5 minutes each.
3. Place specimens in 70 percent acidified alcohol and destain until the specimens are a delicate pink color.

For average-size specimens, destaining is usually accomplished in about 30 minutes. Change the alcohol if it becomes deep red in color.

4. Wash in several changes of 70 percent alcohol, 30 minutes each, until alcohol no longer acquires a pinkish color.
5. Dehydrate in successive changes of 80 and 95 percent alcohol, 30 minutes each.
6. Clear in aniline oil, carbol-xylool or beechwood creosote for 1 hour.
7. Mount in xylo-damar or balsam following method previously described for trematodes.

#### Mounting of Larval and Adult Nematodes

The cuticula of nematodes is quite impervious and the usual methods of fixation and dehydration are not satisfactory for preparation of permanent mounts of these worms. Simple and satisfactory techniques for mounts of this group include mounting in glycerin jelly, glycerin and Langeron's lacto-phenol. In these media, they may be mounted either stained or unstained.

##### *Mounting in Glycerin:*

##### *Materials and Equipment:*

Beakers  
Staining dishes  
Glycerin-alcohol (32)  
Alcoholic eosin (3)  
Slides  
Coverslips, round, 18-22 mm  
Camel's-hair brushes  
Gold size  
Asphaltum

##### *Technique:*

1. Place the specimens in a large beaker and pour a considerable volume of hot glycerin-alcohol over them. Nematodes are frequently mounted unstained, but if it is desired to stain them, a small amount of alcoholic eosin (enough to impart a light pink color) may be added to the glycerin-alcohol after the solution has cooled to room temperature.

2. Place the container in a 37° C. incubator and allow to stand until most of the alcohol has evaporated leaving the specimens in almost pure glycerin.
3. Prepare scrupulously clean slides. Center a slide either on a commercial type turntable or on a block in the head of a small centrifuge. Set the turntable or centrifuge in motion, dip a pointed camel's-hair brush into gold size and lightly touch the slide making a ring the outer dimensions of which about equal the outer dimensions of the coverslip to be used. The ring should be about  $\frac{3}{16}$  of an inch in width. The brush should be lifted before the table or centrifuge stops turning. If the slide is turned too rapidly, the ring will spread and become uneven. If the brush is appressed too heavily against the slide, the ring will be very thick and the cell within will be too small. Ring the slides daily in the same manner until a cell of the desired depth has been made. Two or three applications are usually adequate. Slides should dry 24 hours after the last application of gold size. Just prior to making the mount, apply a fresh ring of gold size to serve as an adhesive for the cover-slip.
4. With a camel's-hair brush, transfer a drop of the glycerin containing the specimens to the center of the cell on the slide. Spread and, if deemed necessary, add more glycerin. A slight excess of glycerin which will ooze out from beneath the margin of the coverslip is desirable in order to prevent entrapment of bubbles. Place the cover-slip on the cell carefully lowering it gradually to a position where the margin of the ring is in contact with the outer margin of the cover-slip. Seal the mount by carefully pressing at several points on the outer margin of the coverslip bringing it into firm contact with the freshly applied gold size.
5. Let slides stand 24 hours to allow time for the gold size to harden. At the end of 24 hours, any slides which show evidence of "leaks" should be set aside and the specimens remounted.
6. The margins of coverslips on slides which are well sealed should be gently wiped with lens paper to remove any excess glycerin, and the slides should be set aside to dry for an additional 24 hours.
7. Again place the slides on a turning device and with a camel's-hair brush apply a ring of asphaltum overlapping the slide and coverslip in such a manner that the preparation is sealed.

## **Permanent Mounts of Helminth Ova**

### **Ringed Slide Technique:**

#### *Materials and Equipment:*

Wire screens, #10, #30, and #60 mesh  
 Formalin, 5 percent  
 Slides, 1 x 3 inches  
 Coverslips, round, 18-22 mm  
 Camel's-hair brushes  
 Asphaltum-gold size mixture (2 parts asphaltum, 1 part gold size)  
 Shellac-gold size mixture (equal parts)  
 Medicine dropper  
 Applicator sticks

#### *Technique:*

1. Mix the fecal specimen thoroughly with a large volume of water and pass successively through #10, #30, and #60 mesh wire screens to remove large particles.
2. Concentrate ova by sedimentation technique described elsewhere and suspend the sediment in 5 percent formalin.
3. Place a clean slide on a turntable or secure it to the head of a small centrifuge and with a camel's-hair

- brush, spin a ring of the asphaltum-gold size mixture. The outside diameter of the ring should be equal to the diameter of the round coverslip to be used. The ring should be about 2 mm in width. The asphaltum-gold size mixture should be about the consistency of glycerin. If it is too thick, thin it with xylol.
4. Set the slide or series of slides aside to dry for 24 hours. If a deeper cell is desired, one or more additional layers may be applied to the ring allowing a 24-hour drying period between each application. Large numbers of slides can be prepared and set aside indefinitely for future use.
  5. At the time the mount is to be completed, an additional fresh ring of shellac-gold size mixture is spun on top of the asphaltum-gold size mixture. The shellac-gold size mixture should be about the consistency of glycerin. If too thick, thin with 95 percent alcohol.
  6. With a medicine dropper, immediately apply a drop of the washed formalin fixed fecal specimen to the center of the cell. A slight excess which will be forced out when the coverslip is appressed to the preparation is desirable.
  7. Touch a round coverslip to one side of the cell and lower it slowly onto the cell. The liquid should spread evenly and cover the entire cell without entrapping any bubbles.
  8. After the coverslip is completely lowered, press the edges down gently with an applicator stick to bring the margin into firm contact with the soft shellac.
  9. Set the slides aside to dry in flat position at room temperature.
  10. The following day, any slides that show signs of evaporation due to incomplete sealing should be dis-

carded. Remaining slides should again be placed on a spinning device and an additional ring of shellac and gold size spun overlapping the margin of the coverslip to seal it to the slide.

11. Following 24 hours or more of further drying, spin a ring of asphaltum-gold size mixture as a final coat.

#### **Double Coverslip Technique:**

##### *Materials and Equipment:*

Wire screens, #10, #30, and #60 mesh  
Formalin, 5 percent  
Medicine dropper  
Coverslips, square, 22 mm  
Coverslips, round, 18 mm  
Slides, 1 x 3 inches  
Permount or clarite  
Applicator sticks

##### *Technique:*

1. Mix the fecal specimen thoroughly with a large volume of water and pass it successively through #10, #30, and #60 mesh wire screens to remove larger particles.
2. Concentrate ova by sedimentation technique described elsewhere and suspend the sediment in 5 percent formalin.
3. Place a drop of the formalized specimen on the center of a 22 mm square coverslip. Cover the specimen with an 18 mm round coverslip.
4. Place 2 or 3 drops of mounting medium on the center of a 1 x 3 inch slide. The exact amount of medium to use is determined by experience.
5. Invert the coverslips so the round coverslip is down and carefully place the preparation on the mounting medium.
6. Press gently around the margin of the square coverslip with an applicator stick to seal the preparation.
7. Dry in flat position until medium has hardened.

### **Glycerin Jelly Mounts:**

#### *Materials and Equipment:*

Wire screens, #10, #30, and #60 mesh  
Glycerin jelly (33)  
Formalin, 5 percent  
Centrifuge tube  
Applicator sticks  
Medicine dropper  
Slides  
Coverslips, round, 18-22 mm  
Scalpel or razor blade  
Camel's-hair brush  
Asphaltum-gold size mixture (2 parts asphaltum, 1 part gold size)

#### *Technique:*

1. Comminute the specimen in a large volume of water and strain successively through #10, #30, and #60 wire screens.
2. Concentrate ova in the fecal specimen by sedimentation and suspend the concentrate in equal parts of warm glycerin jelly and 5 percent formalin in a centrifuge tube.
3. With an applicator stick, mix the suspension thoroughly and set it aside in a 37° C. incubator to

evaporate the formalin. As the formalin evaporates, replace the fluid lost with warm glycerin jelly. This should be done about twice weekly over a period of several weeks.

4. When the ova are suspended in pure glycerin jelly, heat the suspension to about 50° C. and with a medicine dropper, transfer about 2 drops of the suspension to the center of a clean glass slide. While the medium is still warm, apply a coverslip and appress it slowly to the preparation.
5. Set the slides aside at room temperature until the glycerin jelly congeals. With a scalpel or razor blade, cut away any solidified gelatin which may have oozed out from beneath the coverslip.
6. With a camel's-hair brush, apply a coat of asphaltum-gold size mixture over the margin of the cover-slip by spinning the slide on a turning device.



## Chapter 3

# RECOGNITION OF INTESTINAL PARASITES

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### INTESTINAL AMOEBAE OF MAN

Finding and accurately identifying cysts and trophozoites of amoebae in the routine examination of fecal specimens is one of the most difficult tasks confronting the laboratory technician. The intestinal protozoa as a group derive their importance chiefly from the necessity for the differential diagnosis of those which may be of medical importance from those which are not. Since it is often impossible to rely on the clinical picture alone,

the medical officer must rely upon laboratory findings in the diagnosis of many parasitic infections.

There are five species of amoebae which inhabit the human intestinal tract. Various stages as seen in both living condition and in iron-hematoxylin stained preparations will be described in the section which follows. Illustrations are based on iron-hematoxylin stained mounts.

#### ***Endamoeba histolytica***

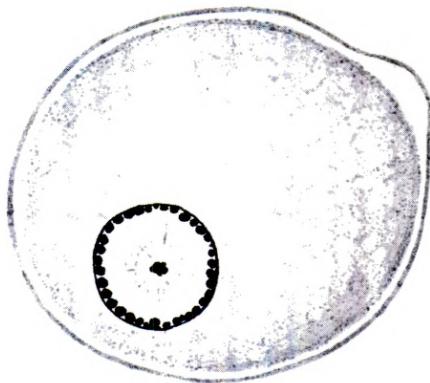
*Trophozoites.* Trophozoites of this species are relatively large, about three or four times the size of a red blood cell. There are two distinct races, a small race and a large race. Smaller strains are not often associated with amoebic dysentery. In living condition about one-third of the amoeba consists of clear, hyaline ectoplasm and the remainder of the organism is finely granular endoplasm in which food vacuoles and the nucleus float about. When warm these amoebae are very active. They tend to travel in a straight line moving rapidly and advancing at either end a single, clear pseudopod, while ingested cells and the nucleus flow and roll about within them as though in a mobile liquid. Food inclusions consist of red blood cells or fragments of other body cells, principally epithelial cells and white blood cells from the intestinal tract. In this species ingested food never includes bacteria, starch, yeast, and the like. The nucleus in fresh preparations may be very indistinctly visible as a faint ring or entirely invisible. In stained preparations the ectoplasm and endoplasm are not differentiated



**Trophozoite**

into clearly defined zones. In all stages of development the nucleus is very nearly perfectly circular. The limiting membrane of

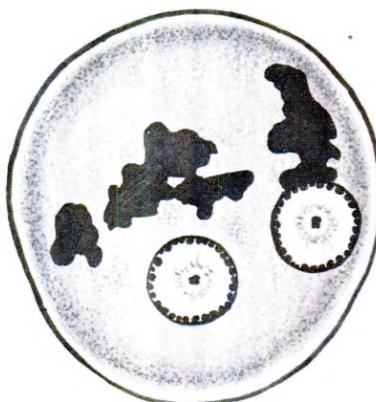
the nucleus known as the nuclear membrane has on its inner lining a thin layer of rather evenly distributed chromatin granules, bead-like in arrangement. At the center of the nucleus is a small dot-like body known as the endosome or karyosome body. In the course of its transition from the active trophozoite to the dormant protected cystic stage several clearly defined stages can be identified.



Pre-cyst



Uninucleate Cyst



Binucleate Cyst

*Pre-cysts.* Progressing from the trophozoite stage the next stage observed is the pre-cyst. This is followed successively by the uninucleate, binucleate, and quadrinucleate cyst or mature form. As the organism commences encystment food inclusions are lost, the ectoplasmic zone becomes much more narrow and only occasional small pseudopods are observed. This is the typical pre-cyst.

*Uninucleate cysts.* As the encystment process advances pseudopods are lost and a well defined cyst wall or protective covering is secreted. At this stage the organism is usually perfectly round or nearly so. Food inclusions are extruded and chromatoid bodies appear, several in number and of varying shapes and sizes. The one-nucleated cyst usually displays a rather broad, oval clear area which is known as the glycogen vacuole.

*Binucleate cysts.* As the organism progresses to the two-nucleate stage the glycogen vacuole becomes smaller or disappears and the chromatoid material begins to congregate into fewer and more uniform-sized masses.

*Mature cysts.* Further division of the nuclei occur resulting in the formation of the mature four-nucleate cyst. Throughout this gradual transition the nuclear characteristics remain unchanged. The dot-like karyosome body and the peripherally arranged bead-like chromatin granules are identical in all stages of development. Glycogen vacuoles are never observed in the four-nucleate cyst. Two principal diagnostic characters identify the mature cyst, namely, four nuclei typical of the species, together with one or two rod-like chromatoid bodies with parallel margins and smoothly rounded ends. In older cysts the chromatoid bodies may be completely lacking. Iron-hematoxylin stained chromatoid material and chromatin granules stain almost jet black.



Mature Cyst

### Endamoeba coli

*Trophozoites.* There are several characteristics which differentiate this species from *E. histolytica* in the living trophozoites. This species is the largest of the intestinal amoebae and displays numerous types of food inclusions including bacteria, starch, portions of discharged body cells and yeast. Rarely red blood cells may occur within the cytoplasm, and then only in situations where other conditions or parasitic infections exist which cause hemorrhaging in the intestinal tract. Movement is sluggish and non-directional. Pseudopods are usually very narrow and composed mostly of endoplasm. The pseudopods are much less hyaline and much shorter and more blunt than those of *E. histolytica*. In unstrained preparations the nucleus is usually visible, appearing as a large refractile ring within which may be seen an eccentric hyaline mass, the karyosome body.

In stained preparations the nucleus of *E. coli* is very characteristic. On the inner periphery of the nuclear membrane the chromatin material is arranged in irregular masses. The karyosome body is large and almost always eccentric or off-center within the nuclear mass. As the organism progresses through various stages in the process of encystment several clearly recognizable transitional forms will be observed. These include

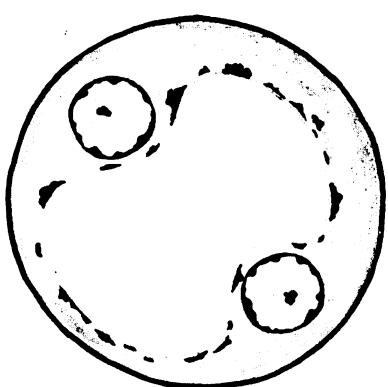


Trophozoite

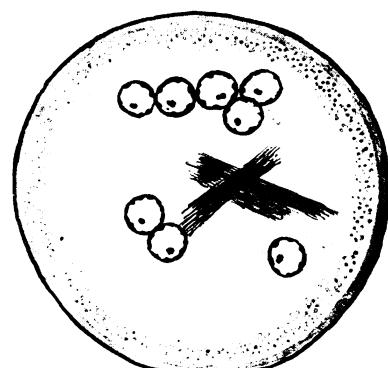
precysts, uninucleate cysts, binucleate cysts, and the mature or eight-nucleated forms. The organism passes through a four-nucleate stage in the process of encystment. This stage is rarely seen, may be easily confused with the mature cyst of *E. histolytica*, and is, therefore, not discussed or illustrated here.



Pre-cyst



Binucleate Cyst



Mature Cyst

*Pre-cysts.* As the organism progresses from the trophozoite to the pre-cystic stage, food inclusions are extruded and numerous small masses of chromatin material appear. Very characteristically about one-third or more of the organism, may at this stage be occupied by a large clearly defined, rather transparent zone, the glycogen vacuole. The vacuole tends to push the nucleus off to one side of the pre-cyst. As encystment progresses a thick elastic cyst wall is gradually secreted about the organism.

*Binucleate cysts.* The next stage observed is that in which the single nucleus has divided resulting in the two-nucleate or binucleate cyst. Frequently in this stage the glycogen vacuole is considerably enlarged and occupies a large proportion of the cyst. The two nuclei are often observed in direct contact with the cyst wall and the large glycogen vacuole forms a characteristic "figure-of-eight" between the two nuclei. This stage of development is very characteristic for *E. coli*. In this stage the area between the glycogen vacuole and the cyst wall is usually filled with numerous irregular masses of chromatoid material.

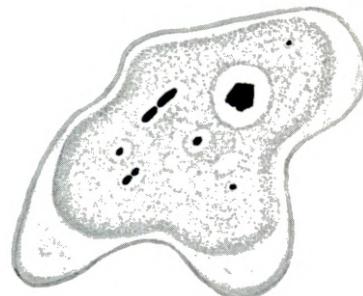
*Mature cysts.* Two further nuclear divisions occur resulting in the eight-nucleated mature form. At this stage most of the chromatoid material has disappeared and the remaining remnants consist of usually not more than two irregularly shaped masses the ends of which are frayed or "splinter-like" and the margins of which are not parallel to each other. Throughout all stages of development the nuclear elements diagnostic of the species remain the same, namely, a large eccentrically placed karyosome body surrounded by a halo of fine granules, and a clearly defined nuclear membrane the inner periphery of which is lined with irregular masses of chromatin material.

## **Endolimax nana**

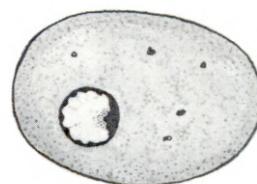
*Trophozoites.* This species is very small, rarely averaging a diameter greater than that of a red blood cell, and occasionally even smaller. Movement is very sluggish and the pseudopods are narrow. Generally, movement is non-directional and pseudopods are extruded in various directions with minimal directional motion. The cytoplasm is usually filled with numerous vacuoles containing bacteria and the cytoplasm is heavily granulated. If the nucleus is visible it can be seen in hazy outline as a rounded hyaline mass. In stained preparations nuclear characteristics, size, and character and abundance of food inclusions, are the principal diagnostic characters by means of which the species can be recognized. The nuclear membrane is often invisible and the nucleus is characterized by a karyosome body which displays an outline much like a "chunk of coal." It is often trapezoidal in shape and may show occasional lobulation. The area between the ill defined nuclear membrane and the karyosome body is a "halo-like" narrow clear zone free of granulation.

*Uninucleate cysts.* As the organism goes into the cystic stage food inclusions are lost and the cytoplasm becomes more granular. Gradually as the transitional stages occur a thin elastic cyst wall is secreted. In the uninucleate stage and subsequent changes as the cysts mature the karyosome body becomes smaller and more dense and the nuclear membrane can be discerned.

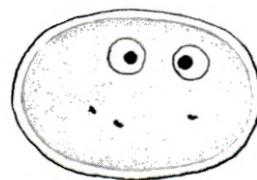
*Binucleate cysts.* In this stage nuclear characteristics are similar to those observed in the one-nucleated form. The nuclei generally lie close together at one pole of the cyst and karyosome bodies are frequently eccentric and in close contact with the nuclear membrane.



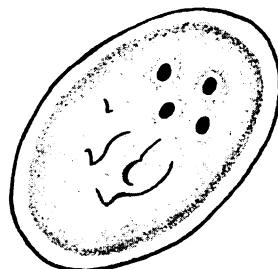
**Trophozoite**



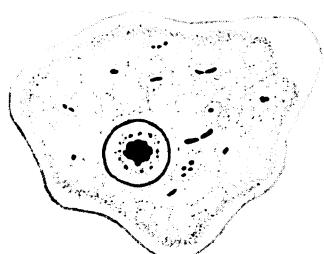
**Uninucleate Cyst**



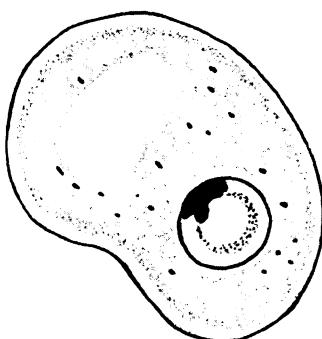
**Binucleate Cyst**



Mature Cyst



Trophozoite



Cyst

*Mature cysts.* The mature four-nucleate stage is the form most frequently seen in routine fecal examinations. In stained preparations the nuclei are generally clustered close together, four in number, give much the appearance of four "eyes" in quadrate arrangement. The karyosome bodies and their surrounding nuclear membranes are oval in shape, the former filling about one-half the total area of the nucleus. Cysts are sometimes round but most often ovoid in shape. Chromatoid bodies are not seen in this species. The endoplasm often contains a number of small refractile granules of a substance known as volutin. Young cysts may also contain small masses of glycogen.

### Iodamoeba butschlii

*Trophozoites.* In the living trophozoites which are about one and one-half times the diameter of a red blood cell, movement is sluggish, pseudopods are leaf-like, greenish-yellow in appearance and broadly clear margined. The trophozoites are easily confused with those of small strains of *E. histolytica*. They are most readily differentiated from the latter by the nature of food inclusions which in the present species consists principally of bacteria.

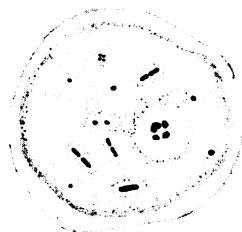
*Cysts.* This is the only species of intestinal amoebae of man in which a single nucleus persists throughout both the trophozoite and cystic stages. A clearly defined pre-cystic stage is not recognized. In the cystic stage the nucleus differs considerably from that of the trophozoite. In both stages the nuclear membrane is distinct. In trophozoites the irregular karyosome body is centrally situated and surrounded by a halo whereas in cysts the karyosome lies at or near the nuclear membrane. The nuclear membrane of cysts in well stained preparations displays irregularly spaced chromatin granules which are margined by a "scallop-like" fringe surrounding the membrane. The cyst is also characterized by a large glycogen vacuole which stains dark mahogany with iodine stain and appears as a clearly defined zone sur-

rounded by a halo in hematoxylin stained preparations. Within the cytoplasm of the cyst there will frequently be observed small dark masses which to the inexperienced observer may be mistaken for bacteria but which are actually inclusions known as volutin granules. These

are similar in consistency to chromatoid bodies seen in other species, but much smaller. Another important characteristic of the cyst is that it is generally neither round nor oval in shape but rather irregular in outline presenting no characteristic form.

### Dientamoeba fragilis

*Trophozoites.* Living trophozoites of this species in fresh preparations extrude clear pseudopods and the movement of the organism is active and progressive. With a drop in temperature the organism soon becomes inactive, rounds up and quickly degenerates. In contact with tap water they quickly rupture and disintegrate. The cytoplasm is frothy granular in appearance and the ectoplasmic and endoplasmic zones are sharply demarcated. Food inclusions generally consist of bacteria. About eighty percent of the organisms of this species observed in routine fecal examinations bear two nuclei. The nucleus consists of an aggregation of separate granules four to eight in number, generally not over five. The nuclear membrane is usually indistinct or often invisible and there is no chromatin lining on the inner periphery of the nuclear membrane. This is the only species of intestinal amoebae of man in which no cystic stage is known, thus far only trophozoites have been observed.



Trophozoite  
(Uninucleate)



Trophozoite  
(Binucleate)

### KEY TO THE TROPHOZOITES OF THE AMOEBAE OF MAN

- |   |                              |
|---|------------------------------|
| 1. Occur in the intestine.....  | 2                            |
| Occur in the mouth.....   | <i>Endamoeba gingivalis</i>  |
| 2. Typical trophozoite with a single nucleus.....   | 3                            |
| Typical trophozoite with two nuclei.....  | <i>Dientamoeba fragilis</i>  |
| 3. Nucleus with comparatively large karyosome and little if any peripheral chromatin.....                                 | 4                            |
| Nucleus with comparatively small karyosome and distinct peripheral chromatin.....   | 5                            |
| 4. Karyosome spherical, usually central in trophozoite nucleus, nuclear membrane delicate and often indistinct.....       | <i>Iodamoeba butschlii</i>   |
| Karyosome often irregular, block-like, and eccentrically placed in nucleus. Nuclear membrane distinct.....                | <i>Endolimax nana</i>        |
| 5. Nucleus with very small, central karyosome and fine peripheral chromatin granules.....                                 | <i>Endamoeba histolytica</i> |
| Nucleus with larger, typically eccentric karyosome and coarse, irregularly distributed peripheral chromatin granules..... | <i>Endamoeba coli</i>        |

## KEY TO THE CYSTS OF THE AMOEBAE OF MAN

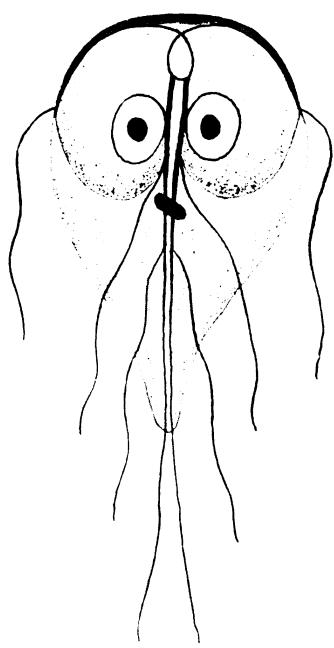
1. Mature cysts with more than one nucleus.....	2
Mature cysts with a single nucleus.....	<i>Iodamoeba butschlii</i>
2. Mature cysts containing four nuclei.....	3
Mature cysts containing eight nuclei.....	<i>Endamoeba coli</i>
3. Nuclei with a very small, dot-like centrally placed karyosome and fine bead-like peripheral chromatin. Chromatoids generally present. Cysts ovoid..	<i>Endamoeba histolytica</i>
Nuclei with large, irregular, block-like karyosome. Peripheral chromatin generally lacking. Chromatoids absent. Cysts elliptical.....	<i>Endolimax nana</i>

## THE INTESTINAL AND VAGINAL FLAGELLATES AND CILIATES OF MAN

The common diagnostic character found in this group of protozoan parasites is that all possess locomotor organs known as flagella. They differ from the amoebae in that their body contours are fairly constant. A number of definite structures known as organelles which have specialized functions occur in the flagel-

lates. These include axostyles which are rigid supporting rod-like organelles, the flagella and undulating membrane for locomotion, and a cytostome or protozoan mouth and gullet, and *blepharoplasts* which function as energy control centers. All organelles are not found within each species within this group.

### **Giardia lamblia**



Trophozoite

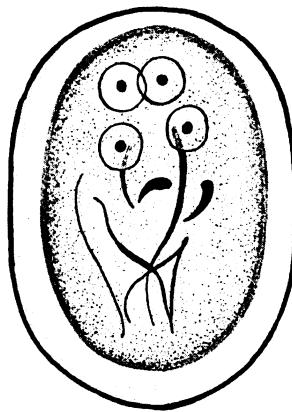
*Trophozoites.* In fresh preparations the trophozoite of this species is actively motile and combines various movements including rotation, rocking, and forward and backward motion in rapid succession. In shape this flagellate resembles a longitudinally cut pear. Its length is more than double that of a red blood cell and its width is slightly greater than the diameter of a red cell. It is shaped from dorsal to ventral surface much like a saucer, being dorsally convex and ventrally concave. The concave side forms an ovoid sucking disc by means of which it is capable of adhering to surfaces.

The detailed morphology of this species is visible only in hematoxylin stained preparations. This is the only bilaterally symmetrical protozoan infecting man. That is to say, right and left halves are identical to each other in the trophozoite and all structures except the central longitudinal supporting structure, the axostyle, are duplicated. Locomotor organs consist of eight flagella, a pair of which arise from anteriorly situated granules known as blepharoplasts. Additionally two pair of flagella originate from the blepharoplasts and are directed backward. A fourth pair

originates at the end of the longitudinal axostyle, the latter forming the framework of skeleton of the organism. In the anterior third of the body there are two nuclei, one on either side of the axostyle. These exhibit clearly defined nuclear membranes and bear prominent ovoid karyosome bodies. Both the karyosome and the nucleus are ovoid in shape. Transversely a little below the center of the organism will generally be seen two rod-like structures lying more or less diagonally across the center of the body. The cytoplasm is finely granular and never contains food inclusions, since this organism obtains its nourishment by absorption of digested food through the body wall.

*Cysts.* In temporary mounts the cysts are readily recognized, particularly if iodine or MIF stains have been added to the preparation. The unstained cysts appear as broadly ovoid symmetrical bodies in which are easily observed four ovoid colorless or hyaline structures with a refractile center, these representing the karyosome body and nucleus respectively. This organism is characterized both in stained and unstained preparations, employing either permanent or temporary stains, by the fact that there is a marked shrinking away of the organism from the thick double walled cysts. The contained organism occupies only about two-thirds of the total area within the cyst.

In hematoxylin stained preparations the nuclei stand out very clearly as four "eye-like" ovoid objects similar in morphology to the nucleus seen in the trophozoite stage. These nuclei are generally grouped, four in a cluster, fairly close to each other in the anterior end of the organism. The remains of the axostyle also appear as a curved or S-shaped rod running down the center of the cyst. Oftentimes delicate fibrils representing the remnants of the flagella observed in the trophozoite stage are also seen in the cyst. When a patient is passing these cysts they are usually found in tremendous numbers although not regularly—they are frequently eliminated from the body in "showers." It is not uncommon to observe several dozen cysts in a low-power field of mag-



Cyst

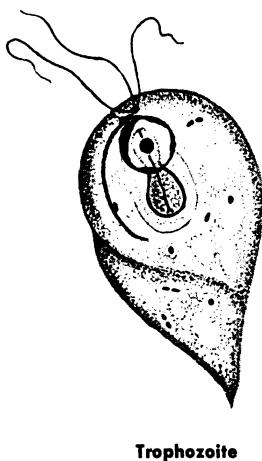
nification. Reproduction in this species very likely occurs first through the process of encystment followed by excystment within the host harboring the trophozoite. Each time this multiplicative process occurs the number of parasites is quadrupled. This is believed to ac-

count for the large numbers of parasites often encountered. Thus encystment serves as both a multiplicative process as well as a process by means of which the organism is protected during periods of adverse environmental conditions.

### **Chilomastix mesnili**

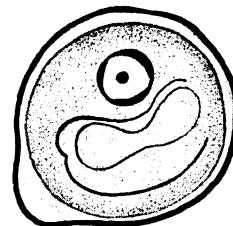
*Trophozoites.* In fresh preparations the trophozoites of this species are very actively motile progressing in a jerky spiral fashion with a directional line of movement. The cytoplasm is faintly greyish-green in color. Movement is accomplished by means of flagella which can be observed only in sluggishly motile trophozoites or in stained preparations.

In hematoxylin stained preparations various external and internal structures are clearly visible. The cytoplasm is finely granular and contains numerous food inclusions, principally bacteria and starch. Other prominent internal structures include the anteriorly placed nucleus and the primitive mouth or cytostome. The cytostome appears as a cleft in the body wall originating anteriorly and extending posteriorly almost one-half the length of the body. This organelle is margined by a short fibril on one side and a longer heavy fibril having the contour of a half "figure-of-eight" on the other. The nucleus is situated in the extreme anterior end and has a well defined nuclear membrane the inner periphery of which is thickened with heavy granules some of which coalesce on one side of the nucleus into a rather broad band. The karyosome body is small, dot-like, and slightly eccentric in position. Immediately above the nucleus in the anterior end of the organism there is a dot-like structure or blepharoplast from which the flagella arise. Three flagella are directed anteriorly and a fourth extends posteriorly about one-half the length of the body. There is no undulating membrane.



Trophozoite

*Cysts.* Stained cysts of this species are ovoid in shape about equal in length to a red blood cell and about two-thirds as wide. The most characteristic morphological structure is a protuberance on one margin giving the cyst a "lemon"-shaped appearance. This protuberance together with a clear ungranulated area beneath and the characteristic shape of the cyst are the most important identifying characters in this stage. Cysts contain a single nucleus situated near the center and bearing a prominent karyosome body and a clearly defined nuclear membrane on which are distributed alternately thin and thickened areas of peripheral chromatin. Remnants of the trophozoite stage contained in the cyst usually include an "hour-glass"-shaped cystostome and remnants of flagella which appear as hair-like fibrils.

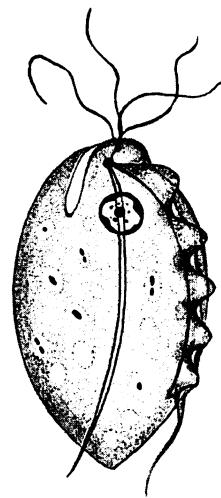


Cyst

### Trichomonas hominis

*Trophozoites.* In fresh preparations this species exhibits a wobbly progressive motion which is brought about by the active vibration of the flagella and the undulating membrane. In order to observe the trophozoite light must be cut down considerably since the organism is very hyaline, clear, and finely granular. The nucleus and other internal structures are not visible in the colorless finely granular cytoplasm.

In hematoxylin stained preparations various internal and external structures are readily observed. The organism is pear-shaped and bears three to five anteriorly directed flagella originating from the blepharoplast, and a single posteriorly directed flagellum which forms the margin of the undulating membrane and terminates in a free flagellum in the posterior end of the body. The prominent undulating membrane is a thin, clear, transparent, fin-like organelle running throughout the entire length of the body. The outer margins of this fold are thrown into numerous ripples. The supporting structure of the organism is the axostyle which originates at the

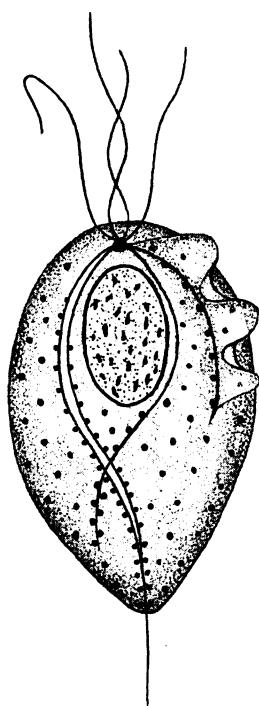


Trophozoite

blepharoplast at the anterior end of the body and runs through its length terminating beyond the posterior margin in a fine point. A primitive mouth or cytostome is present in the anterior third of the body. The nucleus

is prominent and the thin nuclear membrane encloses a clear area bearing several prominent granules. The dot-like karyosome body is eccentric and stands out conspicuously. No cystic stage is known in this species.

### Trichomonas vaginalis



Trophozoite

*Trophozoites.* In fresh urethral or vaginal smears this species is readily visible under high dry magnification. The presence of motile flagellated organisms in discharges from the vagina in females and the urethra in males is diagnostic for this species since no other flagellates occur in the genito-urinary tract of man. This species may also be observed in routine urinalysis on both men and women. The infection is, however, much more common in females.

The cytoplasm is uniformly granular and food inclusions are lacking. Motion is zig-zag and directional. Under reduced light the organisms are highly refractile and their pear-shaped form and characteristic movement is readily observed.

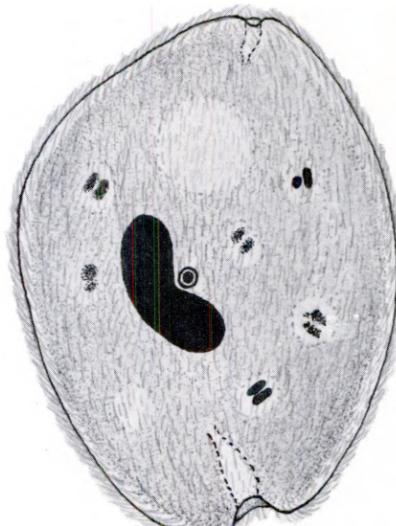
In stained preparations various internal and external morphological characters can be readily identified. There are four anteriorly directed flagella arising from the blepharoplast and a fifth flagellum is directed posteriorly and forms the margin of the undulating membrane. This flagellum terminates as a free fibril about halfway down the lateral margin of the body wall. The nucleus is usually elongated, with a small karyosome centrally situated within the nucleus. The axostyle is relatively slender and curved as it passes alongside of the oval nucleus. The area between the karyosome body and the nuclear membrane is clear. In stained preparations there are numerous rod-like prominent dark staining granules evenly distributed throughout the cytoplasm. These are known as volutin granules and are believed to be the counterpart of chromatoid bodies in other protozoans. The supporting axostyle originates in the blepharoplast and terminates posteriorly in a rather long free portion protruding from the body wall. No cystic stage is known in this species.

## Balantidium coli

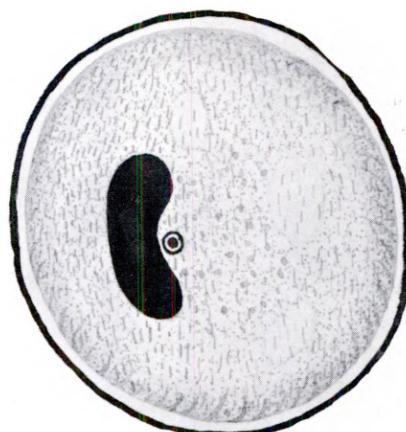
*Trophozoites.* This is the largest protozoan of man. It differs most markedly from the flagellates in the fact that locomotion is by means of cilia. The motile trophozoites in fresh preparations are large, ovoidal, slightly greenish bodies, displaying directional, rapid, smooth gliding movements. Off to one side of the anterior end is a deep cleft which is the cytostome or primitive mouth. The outer surface of the body is covered with a delicate pellicle on the surface of which are implanted in regular rows, numerous short cilia. Food vacuoles are very numerous and consist almost entirely of starch. In living condition one small anterior and a large posterior excretory vacuole can be seen, first enlarging and then expelling their contents at regular intervals.

In stained trophozoites the surface covering of longitudinal rows of cilia can be seen. The most prominent internal structures are the two nuclei, a large kidney-shaped macronucleus in the concave surface of which lies the small dot-like micronucleus. This species is a heavy feeder and the cytoplasm is filled with food inclusions.

*Cysts.* Cysts are not commonly encountered. When seen they are large double-walled spherical bodies eight to ten times the diameter of a red blood cell. The cytoplasm is uniformly heavily granulated and lacks food inclusions. Macro- and micronucleus stain clearly and are identical in outline to those seen in the trophozoite stage.



Trophozoite



Cyst

**Table 1. A Comparative Table of the Medically Important Amoebae of Man**

Diagnostic Characters	<i>Endamoeba histolytica</i>	<i>Endamoeba coli</i>	<i>Dientamoeba fragilis</i>
TROPHOZOITES	Size-----	Quite variable, but generally between 20-30 $\mu$ . There are at least two distinct races based on size. Broadest limits of size range from 15-60 microns.	Quite variable but within the limits of 15-60 $\mu$ .
	Motility-----	One or two blunt pseudopodia are usually present in normal fresh preparations. Movement is rapid and directional.	Less actively motile than <i>E. histolytica</i> . Movement is not directional. Pseudopods form slowly and are short and blunt.
	Cytoplasm and inclusions (in fresh preparations).	Ectoplasm and endoplasm clearly differentiated with former broad and glass-clear. Food inclusions often consist of red cells.	Less hyaline, ectoplasm not clearly differentiated from endoplasm. Food inclusions limited mostly to bacteria, starch and cysts of small protozoans.
	Nuclei in stained preparation.	Nucleus is ring-like, off center in position and indistinct. On the lining of the nuclear membrane is a thin layer of chromatin, beadlike in arrangement. At center of nucleus is a small dot-like karyosome.	Nucleus is ring-like, near center of organism and can almost always be seen in fresh specimens. Karyosome is large and distinct. Chromatin on inner periphery of nucleus in heavy masses.
	Location within host	Commonly the sub-mucosal tissue of the large intestine. Carried by the circulatory system it may infect the liver, lung, brain, and other organs. Occasionally the skin may be involved.	In the lumen of the intestine only. Organism is never invasive.
	Pathogenicity-----	From medical viewpoint this is the most important amoeba of man. With or without clinical manifestations infection with this species is known as amoebiasis. While most infections are asymptomatic a small percentage are clinical.	Non-pathogenic. Of importance only because it is easily confused with <i>E. histolytica</i> .

Table 1. A Comparative Table of the Medically Important Amoebae of Man—Continued

Diagnostic Characters	<i>Endamoeba histolytica</i>	<i>Endamoeba coli</i>	<i>Dientamoeba fragilis</i>	
CYSTS	Size and shape.....	Cysts are formed within the lumen of the intestine. They are roundish and 7–18 $\mu$ in size and are generally smaller than those of <i>E. coli</i> .	Cysts are roundish, 15–20 $\mu$ in diameter.	Cysts are not known in this species.
	Number of nuclei.....	Young cysts, 1 or 2. Mature cysts, 4.	Young cysts 1, 2, or 4. Mature cysts generally 8, sometimes 16.	
	Structure of nuclei and staining properties.	Nuclei as in trophozoites above. Cytoplasm stains weakly with iron-haematoxylin.	Nuclei as in trophozoite above. Cytoplasm stains deeply with iron-haematoxylin.	
	Chromatoid bodies, glycogen vacuoles and other inclusions.	Thick or slender bars with parallel sides and smoothly rounded ends. Chromatoids not always present. Glycogen vacuole is large in uninucleated immature cysts.	Glycogen vacuole large in immature binucleate cysts. Chromatoid bodies not often observed, splinter-like (like small needles), single or in clusters.	
TROPHOZOITES		<i>Ioadamoeba butschlii</i>	<i>Endolimax nana</i>	<i>Endamoeba gingivalis</i>
	Size.....	Trophozoites average 9 to 11 $\mu$ in diameter. Species is usually larger than <i>E. nana</i> .	Very small species averaging 7–9 $\mu$ , often even smaller.	Extremely variable in size. Different authors' measurements range between 6–60 $\mu$ . Average size is between 20 and 30 $\mu$ .
	Motility.....	Movement is sluggish and pseudopods leaf-like in appearance and broadly clear-margined.	Movement is sluggish as in <i>E. coli</i> .	Pseudopods usually broad and rounded, resembling large blisters. Movement is generally rapid in various directions.
	Cytoplasm and inclusions (in stained preparations).	Finely granular and greyish with bacterial inclusions.	Usually contains numerous vacuoles filled with bacteria; appears highly granular.	The endoplasm contains leukocytes, bacteria, tissue cells, food vacuoles and according to some observers, occasionally red cells.
	Location within host.....	In the lumen of the intestine only. Organism is never invasive.	In the lumen of the intestine only.	In the tartar at juncture of teeth with gums, as well as in pus pockets between the gums and the periodontum.

Table 1. A Comparative Table of the Medically Important Amoebae of Man—Continued

	Diagnostic Characters	<i>Ioadamoeba butschlii</i>	<i>Endolimax nana</i>	<i>Endamoeba gingivalis</i>
TROPHOZOITES	Pathogenicity.....	Nonpathogenic.....	Non-pathogenic but cysts are easily confused with those of <i>E. histolytica</i> .	This form may be pathogenic and is often found in mouths of persons suffering from pyorrhea. It should be regarded as a probable pathogen.
CYSTS	Size and shape.....	Usually uninucleate, rarely binucleate.	Young cysts, with 1 or 2 nuclei; mature cysts 4 nucleated, very rarely 8 nucleated.	Cysts are not formed in this species.
	Structure of nuclei and staining properties.	Nuclei as in trophozoite above excepting that more than one mass of chromatin may be present. When this is the case the masses are frequently interconnected by strands.	Nuclei as in trophozoite.	
	Chromatoid bodies, glycogen vacuoles and other inclusions.	Chromatoid bodies lacking. Large glycogen vacuole present which stains dark brown with iodine.	No true chromatoid bodies. Glycogen masses occasionally visible in iodine stained preparations.	

**Table 2. A Comparative Table of the Medically Important Intestinal and Vaginal Flagellates**

Diagnostic Characters	<i>Giardia lamblia</i>	<i>Chilomastix mesnili</i>	<i>Trichomonas vaginalis</i>	<i>Trichomonas hominis</i>
TROPHOZOITES	Size and shape..	About 8 $\mu$ wide by 15 $\mu$ in length. Pear-shaped in dorsal view.	About 10–15 $\mu$ in length and slightly narrower (almost spherical).	Usually 15–18 $\mu$ in length by 5–15 $\mu$ in breadth. Shape variable, usually pyriform.
	Number of flagella.	8	3 anteriorly directed and 1 short posteriorly directed.	4
	Internal morphology.	Two nuclei, two axostyles and two blepharoplasts present. Organism is bilaterally symmetrical. A fused pair of deeply staining curved bars lie diagonally just below center of the organism. The nuclei contain centrally located large deeply staining karyosomes.	Cytoplasm grayish, containing numerous vacuoles and food particles. Round or rarely oval nucleus lies close to anterior end and bears well defined small slightly eccentric karyosome.	Coarse chromatic granules present. Nuclear chromatin in fine granules, without definite karyosome. Ingested bacteria present.
	Undulating membrane.	Absent.	Absent.	Full length of body and ending in a free trailing flagellum.
	Size and shape..	Ellipsoidal in shape. Average length is 9–12 $\mu$ and average breadth is 6–8 $\mu$ .	Lemon-shaped with knob at interior end, rarely oval in shape. Bluish-green in color, averaging about 8 $\mu$ in length.	Cysts are unknown in this species.
CYSTS	Nuclei.....	Usually 4 in number, occasionally 8. Nuclei usually in anterior end of cyst. Numerous inclusions representing remnants of trophozoite structure present.	Single nucleus present. Nuclear chromatin tending to condense into several peripheral masses. Fairly large eccentric karyosome present. Numerous inclusions representing remnants of trophozoite structures present.	

## CESTODES OF MAN (Tapeworms)

Whole specimens or portions of cestodes may be submitted to the parasitology section of the laboratory for species identification. These may be passed by the patient spontaneously or they may be eliminated following the administration of anthelmintics. The technician should be able to identify the eggs, proglottids, and the heads of cestodes, all of which possess important diagnostic characters.

All species of cestodes discharge eggs which, when fertilized and normally developed, contain a motile six-hooked or hexacanth embryo. These eggs may be passed singly, in packets containing several eggs surrounded by a membrane, or within mature gravid proglottids which become detached from the posterior end of the strobila or chain of the main body of the worm. In fresh preparations discharged proglottids are often actively motile.

Morphological characters of attachment organs are very useful in species identification. In the majority of tapeworms organs of attachment consist of various combinations of hooks and suckers. In some species hooks are entirely lacking, while in others there are varying numbers of hooks generally arranged in rows on the anterior-most portion of the

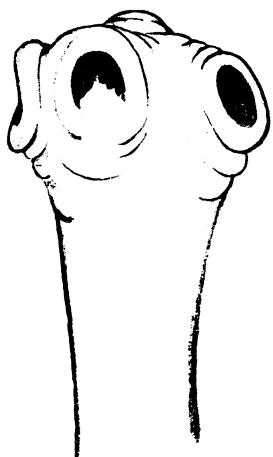
worm, the rostellum. The majority of human tapeworms possess four rounded cupped suckers arranged in a quadrangle at the widest portion of the head.

The principal identification character for species recognition of proglottids is the outline of the egg-filled uterus in gravid segments and the morphology of the male and female reproductive systems in immature segments. Other diagnostic characters include number and location of genital pores, shape of the lateral margins of segments, and the comparative distance between the anterior and posterior and right and left margins of the segment.

With the exception of one species the ova of tapeworms can be identified by variations found within the shell or embryophore. Shells vary in thickness, coloration, and the presence or absence of polar fibrils and striations. With the exception of one species all contain a fully developed six-hooked embryo.

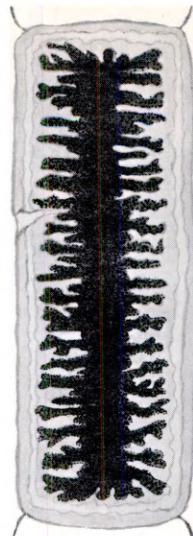
Diagnostic characters for species identification of tapeworms are found in the head, proglottids and eggs. Diagnostic characteristics are described and illustrated in the section which follows.

### **Taenia saginata**

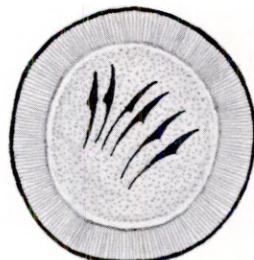


*Adult.* The head of this tapeworm is characterized by the fact that the rostellum is completely lacking and that it bears no attachment hooks. The attachment organs consist of four cup-shaped hemispherical suckers one each at the four corners of the head. Mature proglottids are slightly broader than long while the gravid ones are considerably more narrow and about three

times as long as the broadest portion which is at the center of the segment. In gravid proglottids the central continuous tube of the uterus and its lateral branches, fifteen to twenty in number, are filled with eggs.



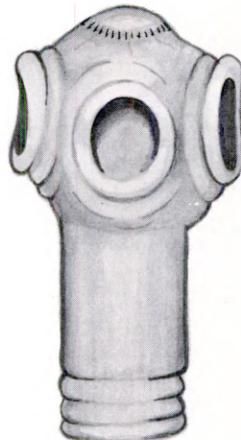
Proglottid



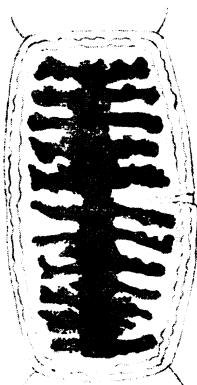
Egg

### Taenia solium

*Adult.* The head bears a prominent rounded rostellum which is armed with a single row of hooks which taper to a fine point and are armed with a lateral denticle at about the middle. Hooks are twenty to thirty-two in number and are alternately large and small in a regular pattern on the margin of the rostellum. The scolex is quadrate in shape and bears at each corner deeply cupped prominent suckers. Mature proglottids are slightly wider than long and contain male and female reproductive systems typical of this genus. The egg-filled and considerably elongated gravid proglottids in the terminal portion of the chain display the characteristic uterine



Head

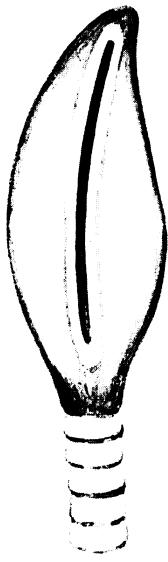


Proglottid

branching diagnostic for this species. The uterus consists of a single longitudinal centrally situated tube from which arise laterally on each side seven to thirteen lateral branches (generally about nine).

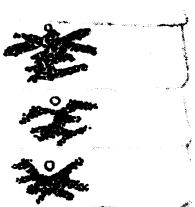
*Eggs.* Taenoid in type. See *T. saginata* above.

### Diphyllobothrium latum



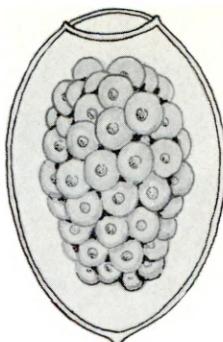
Head

*Adult.* The scolex is small and spoonlike in shape, bearing two longitudinal slits or "bothria" which serve as organs or attachment. Neither hooks nor true suckers are present in this species. Proglottids are very short in the anterior-posterior dimension and about four times as wide as long. In both mature and gravid proglottids the principal diagnostic characters are the centrally situated "rosette" shaped uterus and the genital pore which is in the center of each segment.



Proglottid

*Eggs.* The eggs of this species are broadly ovoidal in shape displaying a contour very similar to that of a hen's egg. The shell is moderately thick, and golden-brown in color when discharged by the patient due to absorption of pigments in the intestinal tract. They contain a partially developed embryo at the time of discharge, generally in about the thirty-two cell stage of development. At its anterior pole the egg bears a prominent cap or operculum and the posterior pole is characterized by a small knob-like protuberance which, however, is not always present.



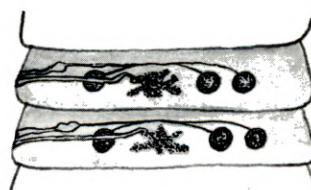
Egg

### **Hymenolepis nana**

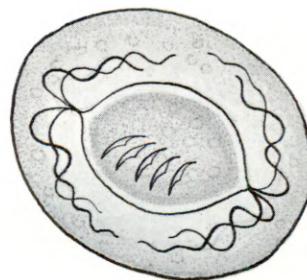
*Adults.* The head is diamond-shaped and bears hemispherical suckers at the four corners. The rostellum is short and armed with twenty to thirty spine-like hooks of equal size arranged in a single ring. The rostellar neck is short and capable of invaginating the rostellum into the head. Either invaginated or evaginated scolices may be seen in routine examinations. In mature stained proglottids the most prominent diagnostic feature which will be observed is the presence of three ovoid irregularly spaced testes along the lower margin. The proglottids are short in antero-posterior dimension and three or four times as wide. Gravid proglottids are completely filled with eggs and the outlines of the uterine branches are obliterated.



Head



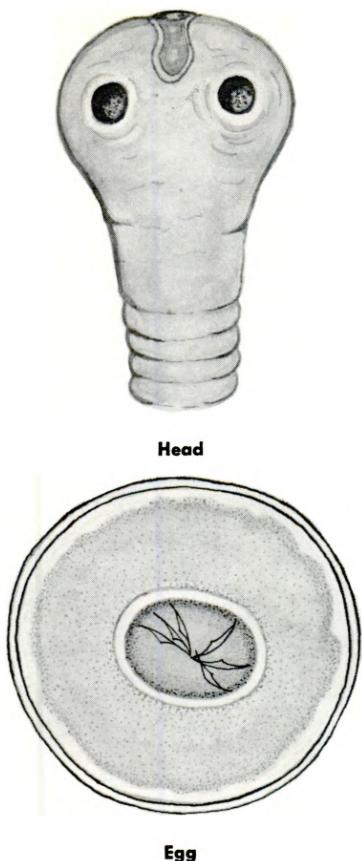
Proglottid



Egg

*Eggs.* The embryophore is very thick and bears prominent knobs at both poles from which arise four to eight filaments which radiate out as fibrils within the egg shell. The area immediately beneath the cortex is highly granular and proceeding inwardly granulation gradually decreases toward the inner periphery of the shell.

### **Hymenolepis diminuta**



*Adult.* The scolex is small and provided with four comparatively small deeply cupped suckers. The rostellum is triangular in shape, unarmed and often invaginated into the scolex. Proglottids are very similar to those of *H. nana* from which they cannot easily be differentiated. In the gravid proglottids the principal diagnostic character is the presence of three irregularly spaced ovoid testes situated along the lower margin. The gravid proglottid is completely filled with eggs obliterating the outlines of the uterus. Proglottids are very short, and about three or four times as wide.

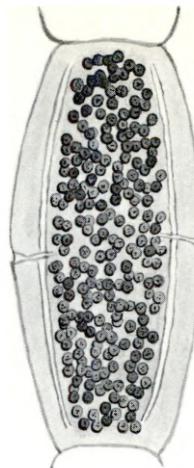
*Eggs.* Eggs are slightly ovoid in shape and covered with a very thick double shell. The inner and outer margins of the protective covering of the embryophore (inner shell) display numerous irregular lobulations. Polar knobs are present but polar filaments lacking.

### **Dipylidium caninum**



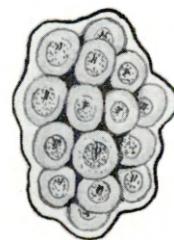
*Adult.* The head is quadrate in shape and bears four deeply cupped suckers and a prominent club-shaped retractile rostellum. The rostellum is armed with one to seven circles of fine spine-like hooks, the number of rows varying with the age of the worm. The anterior spines are the longest and posterior the shortest. Mature proglottids are about as broad as long. As the proglottids mature and become filled with packets of eggs the margins become distended and the segments elongate assuming the shape of a "pumpkin seed." This species is bilaterally symmetrical bearing dou-

ble sets of reproductive organs within each proglottid and genital pores are situated at about the center and on both sides of the segment.



**Proglottid**

*Eggs.* Eggs are generally passed in mother capsules or packets containing varying numbers of eggs up to about twenty. The single shell is relatively thick and sparsely and uniformly granulated throughout. Eggs are usually discharged in packets but occasionally may be observed singly. The most frequent method of passage of eggs from the host occurs through shedding of gravid proglottids from the posterior end of the strobila or chain.



**Egg Packet**

**Table 3. A Comparative Table of the Cestodes of Medical Importance**

Diagnostic Characters	<i>Taenia saginata</i>	<i>Taenia solium</i>	<i>Diphyllobothrium latum</i>
Common name . . .	Beef tapeworm of man . . .	Pork tapeworm of man . . .	Fish tapeworm of man.
Attachment hooks	Unarmed rostellum . . .	Rostellum with double row of attachment hooks.	Lacking.
Attachment organs.	4 suckers . . .	4 suckers . . .	2 longitudinal slits or bothria.
Length . . .	12–36 feet . . .	12–14 feet . . .	As long as 50 feet.
Number of proglottids.	As many as 2,000 . . .	1,000 or less . . .	300–4,000.
Recognition characters of sexually mature proglottids.	Gravid proglottid elongated with uterus consisting of 15–30 dichotomous (tree-like) branches. Genital pores irregularly alternating on margin of each proglottid.	Gravid proglottid elongated with uterus consisting of 12 or fewer dendritic branches. Genital pores irregularly alternating on margin of each proglottid.	Gravid proglottid narrow in anterior-posterior dimensions and containing a "rosette-like" uterus at center of segment. Genital pore present on each proglottid on mid-ventral line.

Table 3. A Comparative Table of the Cestodes of Medical Importance—Continued

Diagnostic Characters	<i>Taenia saginata</i>	<i>Taenia solium</i>	<i>Diphyllobothrium latum</i>
Ova.....	Approximately 30 x 52 $\mu$ in size round or ellipsoidal with heavy radially striated shell and containing a 6-hooked hexacanth embryo.	Indistinguishable from those of <i>T. saginata</i> .	Ova large averaging 45 x 70 $\mu$ elliptical, brown in color and provided with an operculum.
Primary host.....	Man.....	Man.....	Man, dog, wolf, bear and other carnivores.
Intermediate host.....	Normally cattle.....	Normally hogs.....	Primary intermediate hosts are minute crustaceans. Secondary intermediate hosts are various fresh water fish.
Cycle in brief.....	Expelled single proglottids or ova scattered on soil with feces. When taken up by cattle liberated hexacanth embryo bores through intestinal wall into circulation where it is carried to intermuscular connective tissue. In muscle tissue it encysts and becomes a cysticercus.	Terminal proglottids frequently discharged in chains (ova may be expelled singly) taken up by hogs. In alimentary tract of hog, hexacanth embryo bores through intestinal wall and is carried by circulation to intermuscular connective tissue where cysticercus develops in 3 or 4 weeks. When infective cysticerci are taken up by man the worm matures in about 3 months.	Discharged eggs, after period of development in water, liberate ciliated embryo or coracidium which is taken up by crustacean where first stage of larval development occurs. If infected crustacean is eaten by any one of several species of fish the larva works its way out of alimentary tract and lodges in the muscles or viscera becoming infective plerocercoid. Development is completed when plerocercoid is taken up by suitable primary host.
Remarks.....	Cysts survive 6 weeks in meat refrigerated at 32° F. and about 6 days in meat kept at 15° F. Heating to 159° F. kills cysts in 5 minutes.	Principal danger in case of this species of tapeworm is that autoinfection may occur, i.e., man may serve as the intermediate host. Chief methods of control are through cooking of pork and effective treatment of human cases.	Plerocercoids are killed by cooking fish for 10 minutes.
Diagnostic Characters	<i>Hymenolepis nana</i>	<i>Hymenolepis diminuta</i>	<i>Dipylidium caninum</i>
Common name.....	Dwarf tapeworm of man.....	Rat tapeworm.....	Double-pored tapeworm of dogs and cats.
Attachment hooks.....	Rostellum armed with single row of hooks.	Rostellum small, unarmed.....	Retractile rostellum with 3 or 4 rows of easily detached hooks.
Attachment suckers.....	4 suckers.....	4 suckers.....	4 rounded suckers.....

Table 3. A Comparative Table of the Cestodes of Medical Importance—Continued

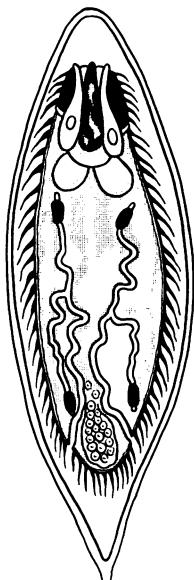
Diagnostic Characters	<i>Hymenolepis nana</i>	<i>Hymenolepis diminuta</i>	<i>Dipylidium caninum</i>
Length.....	Approximately 2 inches.....	8-24 inches.....	4-16 inches.
Number of proglottids.	100-200.....	Up to 2,000, occasionally even more, trapezoidal in shape.	Usually 50 or less.
Recognition characters of sexually mature proglottids.	Mature ripe proglottids much broader than long. Three testes present and arranged in a transverse row across the proglottid.	Wide, narrow in anterior-posterior dimensions. Genital pores on one side only. Three testes, two on one side of midventral line and one on the other.	Gravid proglottid markedly constricted at anterior and posterior extremities, in shape of a cucumber or pumpkin seed. Genital pores on each side at center of each bilaterally symmetrical proglottid.
Ova.....	Eggs elliptical with two membranes, the inner one of which has polar knobs from which thread-like filaments arise. Ova contain hexacanth embryo.	Ova similar to those of <i>H. nana</i> , larger, 60 x 80 $\mu$ with double shell, the inner wall of the outer shell distinctly undulated.	Ova almost spherical 45 x 50 $\mu$ with shell consisting of two thin layers. Ova contains hexacanth embryo.
Primary host.....	Generally rats and mice. Most common tapeworm of man in United States.	Generally rats and mice and occasionally man.	Generally dogs and cats. The species is an incidental parasite of man.
Intermediate host.....	No intermediate host required. Entire cycle can be and generally is completed in one host.	Various insects including larval fleas and certain species of cereal infesting beetles.	Various insects including principally larval fleas.
Cycle in brief.....	Embryonated eggs ingested with food or drink or from soiled fingers liberate hexacanth embryo which bores into intestinal mucosa where it transforms into minute cysticercoid in about one week. Cysticercoid drops back into lumen of intestine and develops into mature worm in about 3 weeks.	Expelled ova are ingested by suitable insect intermediate host and develop into infective cysticercoids. When insects containing infective cysticercoids are swallowed they evaginate the scolex, attach and mature.	Expelled ova eaten by suitable insect intermediate host develop into infected cysticercoids. When infected insects are swallowed the cysticercoid evaginates the scolex, attached and matures.
Remarks.....	Experimentally the cysticercoid will develop in certain beetles and fleas but intermediate host is not essential in life cycle of this parasite.	Man is an incidental host of this parasite. Development takes place in primary host in about 3 weeks.	A large percentage of dogs are infected. Infected animals should be promptly treated. Majority of human infections occur in children.

## TREMATODES (flukes)

Various species of flukes parasitize different organs of the human body including the lungs, liver, intestine, and certain blood vessels in the mesenteries and bladder. Because of their location within the human host, adults are rarely eliminated from the body and adult

morphology will not, therefore, be considered here. Depending upon the organs in which adults localize their eggs may be recovered from sputum, feces, or urine. Characteristic morphology of trematode ova will be described and illustrated in the section which follows.

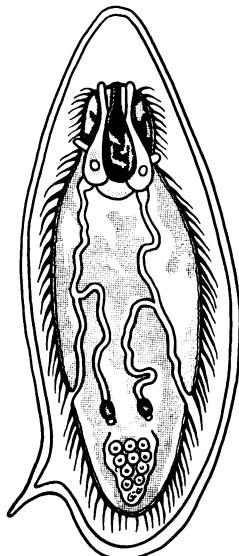
### **Schistosoma haematobium**



Egg

*Eggs.* Eggs are spindle-shaped, rounded anteriorly and conical at the posterior extremity which is tipped terminally with a delicate, relatively blunt-pointed spine. The egg is nonoperculate, bears a thin transparent single shell, is yellowish-brown in color and normally contains a fully developed ciliated motile miracidium when passed. In older infections many eggs will be in various stages of disintegration.

### **Schistosoma mansoni**



Egg

*Eggs.* Eggs are narrowly rounded anteriorly and more broadly rounded posteriorly. The most prominent diagnostic character is the presence of a long, sharp, lateral spine in the posterior third of the egg. Eggs are transparent, bear a thin single nonoperculate shell, are yellowish-brown in color, and normally contain a fully developed ciliated motile miracidium. Eggs in various stages of disintegration often occur.

### **Schistosoma japonicum**

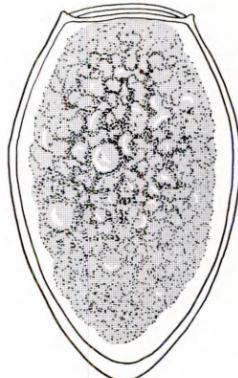
*Eggs.* Eggs are nearly ovoidal in contour, and only slightly longer than broad. At the side near one end there is a depression from which arises a small incurved spinose process which resembles a small recurved hook. This process can be seen only when the egg is properly oriented. The shell is thin, nonoperculate, single, pale-yellow in color, and normally developed eggs contain a ciliated motile miracidium. Disintegrated eggs often occur.



Egg

### **Paragonimus westermani**

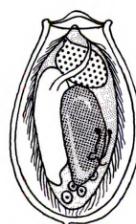
*Eggs.* Eggs are moderately large, broadly ovoidal, brown in color and capped with a broad, flattened only slightly convex, operculum which is ringed by a raised marginal ridge giving rise to some shouldering. Posteriorly the shell is thicker than in the anterior half. The eggs which are generally passed in the sputum are occasionally swallowed and passed in the feces. Eggs are undeveloped when eliminated from the host and closely packed with yolk cells. There is a broad clear zone between the clustered yolk cells and the shell.



Egg

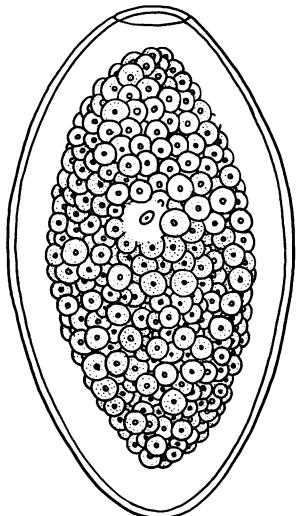
### **Clonorchis sinensis**

*Eggs.* Eggs of this species are relatively thick-shelled, operculate, and are among the smallest eggs passed by man. They are light brown in color, ovoidal in shape, and the operculum rests within a rimmed depression the margins of which form pronounced shoulders which do not follow the contour of the shell curvature. The posterior end of the egg is thickened and bears a small median protuberance which is oftentimes recurved and comma-shaped in lateral view.



Egg

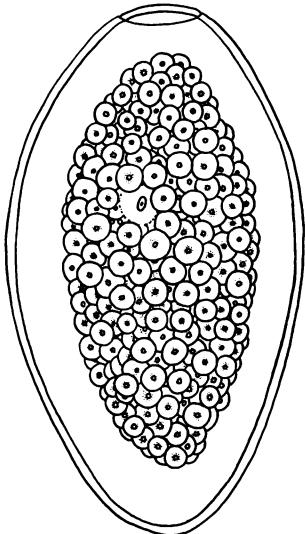
### **Fasciolopsis buski**



Egg

*Eggs.* Eggs are very large, possess a clear thick shell, are goose egg-shaped, and bear a small convex operculum the contour of which is continuous with the shell. They are undeveloped when passed from the host and packed with yolk cells in which the granules are evenly distributed throughout. There is a broad clear zone between the shell and the mass of yolk cells.

### **Fasciola hepatica**

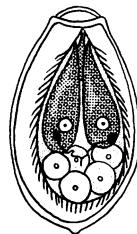


Egg

*Eggs.* Eggs closely resemble those of *F. buski*. They can be differentiated from the latter species by the distribution of yolk cell granules which in the present species are clustered about the yolk cell nuclei. They also tend to be deep golden-brown in color due to absorption of bile pigment.

### **Metagonimus yokogawai**

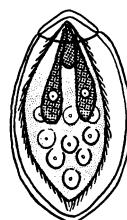
*Eggs.* Eggs are thick-shelled, yellow-brown in color, operculate and contain a fully developed bilaterally symmetrical miracidium when they are discharged by the host. The operculum is relatively broad and is set on top of a rim and not within it as in the closely related species *C. sinensis*. The shoulder at the point of junction of the operculum with the remainder of the shell is not prominent and the contour of the operculum is almost smoothly continuous with the shell.



**Egg**

### **Heterophyes heterophyes**

*Eggs.* Eggs are operculate, very small, brownish in color and contain a well developed bilaterally symmetrical ciliated miracidium. Eggs are difficult to differentiate from those of *C. sinensis* and *M. yokogawai*. Ova can be separated from those of *C. sinensis* by the nature of attachment of the operculum which in this species is set upon and not within a rim.



**Egg**

**Table 4. A Comparative Table of the Medically Important Schistosomes (Blood Flukes) of Man**

Diagnostic Characters	<i>Schistosoma haematobium</i>	<i>Schistosoma mansoni</i>	<i>Schistosoma japonicum</i>
Geographical distribution.	Apparently limited to the African continent but widespread there.	Eastern and central Africa, Caribbean area, and Northern South America into Brazil.	Numerous heavily infested endemic areas in China, Japan, and the Philippine Islands.
Morphology-----	Male is cylindrical, 12-15 mm. in length and not exceeding 1 mm. in width. Oral and ventral suckers close together and in anterior end of the worm. Alimentary canal forks into two branches just posterior to the ventral sucker but unites again to form a single tube down the center of the posterior half of the body. Females are cylindrical and thread-like, exceeding males in length. Ovary elongate lying anterior to the intestinal tube. Vitelline glands occupy posterior end of the body and lie lateral to the intestine. Uterus consists of a long tube opening to the exterior through genital pore which is immediately posterior to ventral sucker. The female lies within gynecophoric canal of male.	Adults closely resemble those of <i>S. haematobium</i> . Male is about 12 mm. long, possesses 6-9 testicular tubules and the short intestinal trunks unite into a single blind pouch which is longer than that of <i>S. haematobium</i> . Body of the male is flattened and folded ventrally to form a canal in which female is held by the male. Vitelline glands occupy the posterior two-thirds of the body. Ovary lies in anterior third of the body and at that level the two intestinal trunks converge to form a single blind pouch.	Adults resemble those of the other two important Schistosomes of man. Male is 12-20 mm. long and 0.5 mm. in diameter. Integument is covered with small spines more prominent near the suckers and the gynecophoric canal. There are seven testes. Female about 26 mm. long and integument also covered with spines. Ovary is just a little behind center of body. Vitelline glands limited to lateral margins of posterior quarter of the body. Uterus consists of long straight tube containing up to fifty eggs at one time.
Primary intermediate host(s) in nature.	Man only-----	Man only-----	Man and other mammals including dogs, cats, horses, cattle, rats, mice, and many others.
Secondary intermediate host(s).	Snails of the genus <i>Bulinus</i> commonly and two other genera rarely.	Snails of the genus <i>Planorbis</i> .	Snails of the genus <i>Oncomelania</i> .

**Table 4. A Comparative Table of the Medically Important Schistosomes (Blood Flukes) of Man—Continued**

Diagnostic Characters	<i>Schistosoma haematobium</i>	<i>Schistosoma mansoni</i>	<i>Schistosoma japonicum</i>
Life cycle-----	Coupled adult worms lodge in the finer veins of the primary host, especially in the bladder and uterus, and the female alone migrates into the finer blood vessels and deposits her eggs in the venules. Eggs by means of their sharp points and lytic secretion force their way through the tissue into the bladder from which they are expelled by the primary host with urine. Occasionally they may be discharged with the feces. Ova contain well developed miracidium and in contact with water the miracidium quickly escape by rupture of the shell after which they seek appropriate snail host. In the latter, two generations of sporocysts are produced. Daughter sporocysts produce the infective fork tailed cercariae (sometimes called furcocercariae) which actively penetrate the skin of man and enter the circulation to lodge in the venules of the bladder, uterus, and sometimes the intestine.	Coupled adult worms lie in the small venules in the wall of the large intestine. Female alone migrates to the end of these venules and packs them with eggs. Eggs by lytic action and by means of their spinous process penetrate out of the small blood vessels and tissues eventually reaching the lumen where they are expelled with feces. Ova that do not reach the intestine soon disintegrate in the blood vessels. On contact with water ciliated miracidium is liberated and seeks out and penetrates appropriate snail. In the snail two generations of sporocysts are produced, the second of which gives rise to the fork tailed cercariae. These escape from the snail and on contact with the skin of man they penetrate and gain entry to the circulatory system and make their way to the small vessels of the large intestine completing the cycle.	Coupled adult worms lie in the smaller venules in the mesentery of the small intestine and frequently within the muscular and mucous layer of that portion of the alimentary tract. Female migrates to the very small branches of the blood vessels and deposits eggs singly packing the venules one at a time. By pressure and the secretion of lytic substances eggs rupture through into the lumen and are expelled with feces. In water, ciliated miracidium ruptures the shell of the egg and swims freely. On contact it invades suitable intermediate snail host. In the snail, sporocysts and daughter sporocysts are produced. Second generation sporocysts produce the cercariae which on contact with suitable primary host enter by active penetration of the skin and reach the circulation through which they are carried to the blood vessels in the region of the small intestine where they grow to maturity thus completing the cycle.
Ova (all very characteristic and readily differentiated).	Large, oval in shape, nonoperculate, measuring about $60 \times 160\mu$ tipped at the extreme posterior extremity with a short, sharp spine.	Moderately large, oval in shape, nonoperculate, tipped marginally near the posterior extremity with a sharp lateral spine.	Smaller than the other species of schistosomes, measuring about $45-60\mu$ and bearing near posterior end and to one side of mid-line a wart-like thickening where an abbreviated spine is seen when ova is lying on its more flattened surface.

**Table 4. A Comparative Table of the Medically Important Schistosomes (Blood Flukes) of Man—Continued**

Diagnostic Characters	<i>Schistosoma haematobium</i>	<i>Schistosoma mansoni</i>	<i>Schistosoma japonicum</i>
Effect on host . . . . .	Penetration of the cercariae often produces severe itching and frequently a definite rash persisting for several days. Secondary infection may occur at points of penetration. In later stages of the disease, symptoms are due largely to movement of the eggs in the mucous membranes of the bladder where they may cause inflammation, cause growth to be formed, and lacerate the blood vessels resulting in bloody urine and acute pain on urination. Symptoms in infections of long duration are due largely to movement of ova in the general circulation and their lodgement in various organs, especially the liver.	Disease produced by infection with this species is known as intestinal schistosomiasis. This infection gives rise to dysenteric symptoms, bloody mucous and in older chronic cases to the formation of polypoid growth within the bowel, latter being due to the proliferation of tissue about masses of disintegrated ova which fail to break through into the intestinal lumen.	Causes "Katayama" disease. There are three distinct stages in course of the disease including period of incubation, period of egg laying and period of proliferation and tissue repair, the latter being chronic stage. Latter stage is characterized by great enlargement of the liver and spleen, by intestinal disturbances, thickening of intestinal wall and formation of ulcers and polypoid growths.

**Table 5. A Comparative Table of Medically Important Trematodes Other Than Schistosomes**

Diagnostic Characters	<i>Clonorchis sinensis</i>	<i>Fasciolopsis buski</i>	<i>Paragonimus westermani</i>
Geographical distribution.	Most important liver fluke of man in parts of China, Japan, Formosa and Indo-China.	Common in India, China, Formosa and Indo-China.	Common infection of man in Asia. A very similar or identical fluke is found in cats, dogs, pigs, and wild carnivores in Africa and North and South America.
Morphology of adult.	Flat, relatively small 10-20 mm long and 2-4 mm wide. Two suckers are present, the oral being larger, and the acetabulum is situated about one-third length of body from anterior end. Testes (two) situated in posterior end, greatly branched.	Largest and best known of the intestinal flukes of man. Worm is leaf-like in appearance, measuring over an inch in length and about a half inch in width. Ventral sucker which lies close to the anterior end, is very large and three to four times the size of the oral sucker. Ovary branched at about middle, testes posterior.	Almost round in cross section about 12 mm broad and one-third as wide, reddish brown in color. Surface is covered with large spines of various forms. Caeca elongate, flexuous, unbranched. Ovary compact, just posterior to prominent ventral sucker.
Primary host(s)	Man, cat, dog, mink, pig, and several other mammals.	Man and pigs commonly; dogs occasionally.	Man, possibly the same species also infecting cats, dogs, pigs and various wild carnivores.
Secondary host(s)	Snails of the genus <i>Bithynia</i> and certain species of fish.	Snails of the genus <i>Planorbis</i> and <i>Spirorbina</i> .	Snails of the genus <i> Melania</i> and certain crustaceans.
Development	Operculate egg passes with feces from primary host and in water liberates ciliated miracidium which enters suitable snail host. In the snail the miracidium loses its cilia and becomes the sporocyst. Within the sporocyst germinal cells give rise to motile rediae which migrate to the hepatopancreas. In that organ the rediae produce either daughter rediae or cercariae. Cercariae push their way through the tissues of the snail and swim freely in water. Upon reaching a suitable fish they enter and develop to infective metacercariae in the muscles.	Operculate ova hatch within two or three weeks after discharge and liberated miracidium seeks appropriate snail intermediate host. Development in the snail is similar to that of <i>C. sinensis</i> . Liberated cercariae encyst on water chestnut or water caltrop. Human infections result from the habit of cracking water caltrop and water chestnuts with the teeth.	After usual cycle in the snail, knob-tailed cercariae emerge and penetrate the thinner chitin of certain fresh water crabs and crayfish, losing their tails and encysting in the muscles as infective metacercariae. In suitable primary host ingested metacercariae are freed in the small intestine where they penetrate out into the thoracic and abdominal cavities and in about 30 days pass through walls of the lung into the bronchioles.

**Table 5. A Comparative Table of Medically Important Trematodes Other Than Schistosomes—Continued**

Diagnostic Characters	<i>Clonorchis senensis</i>	<i>Fasciolopsis buski</i>	<i>Paragonimus westermani</i>
Ova-----	Small, ovoid, dark-colored, operculate, about 15–30 $\mu$ , may remain in water as long as five weeks before hatching.	Operculate, large, measuring about 80 x 125 $\mu$ ; yolk granules clustered around yolk cell nuclei.	Relatively large, approximately 60 x 100 $\mu$ .
Effect on host----	Presence of parasites causes marked proliferation of the epithelium and connective tissue of the liver. Heavy infections may cause cirrhosis of the liver and death of the patient.	Causes considerable gastrointestinal disturbance. Heavy infections cause anemia and in very heavy infections, emaciation is a common symptom.	May cause a chronic bronchial cough and bloody sputum suggestive of tuberculosis.
Diagnostic Characters	<i>Heterophyes heterophyes</i>	<i>Metagonimus yokogawai</i>	<i>Fasciola hepatica</i>
Geographical distribution.	Found in Egypt, Palestine, central and south China, Japan, Korea, Formosa and the Philippine Islands.	Common parasite of man in Egypt, Japan, China, Korea and Formosa.	Cosmopolitan in distribution, particularly abundant where domestic sheep and cattle are raised. Found in North and South America, Europe, Asia and Africa.
Morphology of adult.	Very small, approximately 0.5 x 1.5 mm. Oral sucker small, or ventral surface of the body. Ventral sucker about three times as large as oral sucker located adjacent to the ventral sucker and bearing numerous spines.	Very small, about 0.5 x 2.0 mm, roughly triangular in shape about two times as long as wide in side to side dimensions. Anterior extremity narrow and covered with spines. Ventral sucker larger than oral sucker and to one side of median line. Testes lie in posterior end of body, somewhat symmetrical.	Excretory, digestive and reproductive systems well developed. Hermaphroditic (both sexes in same organism). Male system consists of two many-branched testes in posterior end of worm connected to seminal receptacle by vas deferens. Seminal fluid passes to region of genital pore through the ejaculatory duct. Female system consists of two ramified ovaries in lower anterior third of organism which connect by means of oviduct to the uterus. Yolk glands on lateral margins empty into uterus through vitelline duct.
Primary host(s)---	Man, dogs, cats, rabbits and several other mammals.	Man and several other mammals.	Cattle, sheep, and man. Over 300 human cases are on record.

**Table 5. A Comparative Table of Medically Important Trematodes Other Than Schistosomes—Continued**

Diagnostic Characters	<i>Heterophyes heterophyes</i>	<i>Metagonimus yokogawai</i>	<i>Fasciola hepatica</i>
Secondary host(s)	Snails of the genus <i>Pironella</i> serve as first intermediate hosts and fish as secondary intermediate hosts.	Snails of the genus <i>Melania</i> serve as first intermediate hosts and fish as secondary intermediate hosts.	Snails possessing lungs, belonging to the genus <i>Limnaea</i> .
Development	Adults live in small intestine of the primary host frequently in large numbers. Ova are completely embryonated when they leave primary host. Miracidia hatch soon after contact with water and seek snails of the genus <i>Pironella</i> . After usual development in snail liberated cercariae penetrate beneath scales of certain fish where they encyst as the infective metacercariae.	Infective metacercariae are liberated from their cysts in the duodenum where they burrow into the intestinal mucosa and grow to maturity and deposit eggs. The eggs break out into the lumen and pass out with the feces. They may also break out of intestinal wall into the general circulation.	Poorly developed eggs excreted in feces, develop in water and liberate free swimming miracidium which penetrates snail and lodges in lung tissue where it transforms into the sporocyst. Within sporocyst rediae are produced and they migrate to the hepato - pancreas and produce either daughter rediae or cercariae. The single tailed cercariae escape from the snail, and encyst on vegetation as metacercariae. On being taken by definitive host, they are liberated in the intestine, grow to maturity and then by way of the bile ducts they migrate to the liver.
Ova	Operculate, small, brownish in color, containing well developed ciliated miracidium. Difficult to distinguish from those of <i>C. sinensis</i> and <i>M. yokogawai</i> .	Operculate, small, brownish in color, containing well developed ciliated miracidium. Difficult to differentiate from those of <i>C. sinensis</i> and <i>H. heterophyes</i> .	One of the largest of helminth eggs, ovoid in shape and about 80 x 150 $\mu$ yellowish-brown in color, operculated. Eggs immature when discharged, development taking place in water.
Effect on host	Of little pathogenic importance in either man or animals.	In very heavy infections persistent intestinal disturbances and diarrhea are present. Ova deposited into the general circulation reach the heart and central nervous system, producing cardiac and nervous disturbances.	Moderate to heavy infestations in man give rise to severe symptoms. Patient loses weight and becomes weak and anemic. In animals there are marked liver changes including fibrosis in well established infections. The disease in animals is known as "liver rot".

## NEMATODES (roundworms)

Roundworms constitute the most common parasites of man throughout the world. In tropical and semi-tropical regions infection with one or more nematodes is acquired very early in childhood and persists throughout life. Among poorly sanitized native populations in the tropics these infections are universal. Roundworm infections are not uncommonly encountered among our troops stationed in regions of the world where a high reservoir of infection exists in the indigenous population.

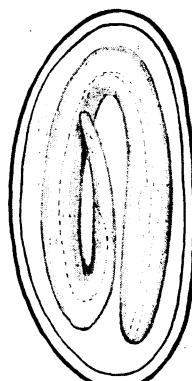
Laboratory diagnosis of roundworm infection is based upon recovery and identification

of eggs, larvae, or adults depending upon the particular species harbored by the patient and the stage of development in which infective stages are eliminated. In freshly passed stools adults are usually intact since their bodies are fairly rigid, unsegmented, and the outer covering or cuticula is quite resistant to bacterial action and decay. In order to observe morphological characters the worms should be processed and mounted preparations made according to techniques described elsewhere in this manual.

### ***Enterobius vermicularis* (pin-worm)**



Adult Female



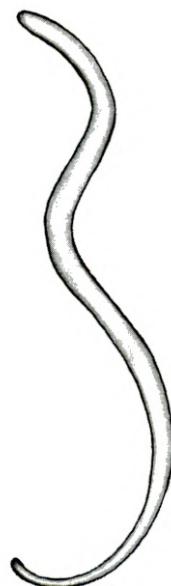
Egg

*Adults.* Adults are ivory white in color, rigid, bristle-like, with the posterior third of the body attenuated and terminating in a fine rather sharp point. The organism does not exceed one-half inch in length. The head is slightly expanded and bears three labia in a triangular pattern as observed in direct anterior view. The long muscular esophagus terminates in a distinct bulb.

*Eggs.* Eggs are flat on one side and broadly rounded on the opposite side, their contour being suggestive of loaves of bread. The shell is moderately thick and consists of three layers, a thick outer albuminous layer, an inner thinner hyaline layer and a membrane which immediately surrounds the embryo. The shell is clear and colorless and eggs normally contain a fully developed motile larva when passed. Eggs are encountered infrequently in routine examinations but in suspected infections they are readily recovered by use of any of several special techniques described elsewhere in this manual.

### **Ascaris lumbricoides (large roundworm of man)**

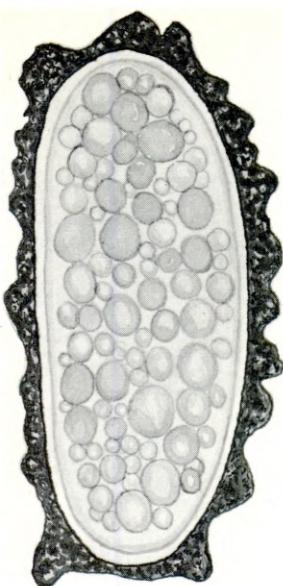
*Adult.* This is the largest roundworm which infects man. Fully mature females may attain a length of eight inches. When freshly passed they are light flesh colored in appearance and quite relaxed presenting much the appearance of an earthworm. The anterior extremity is blunt-pointed and the organs of attachment consist of three fleshy lobes arranged in a triangular pattern when observed in end view. The posterior extremity is tapered and more finely pointed. Males are about half as large as females and the posterior extremity is re-curved sharply and bears two spicules on the inner margin of the curvature. Spontaneous elimination of adult worms is not uncommon. In children these may be regurgitated with vomitus or the worms may actively migrate from the stomach through the esophagus and creep out the mouth or nostrils. Any heavy bodied roundworm in excess of a few inches in length which is eliminated by humans can be stated with certainty to be either *Ascaris lumbricoides* or the closely related and morphologically indistinguishable *Toxacara canis*, normally found in dogs but which may also infect man, particularly children.



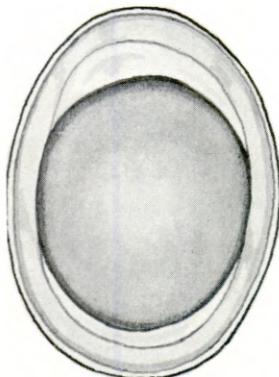
**Adult Female**



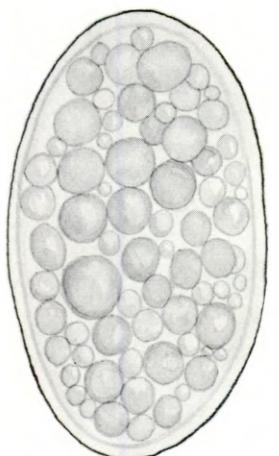
**Normal Corticated Fertilized Ova**



Corticated Unfertilized Ova



Decorticated Fertilized Ova



Decorticated Unfertilized Ova

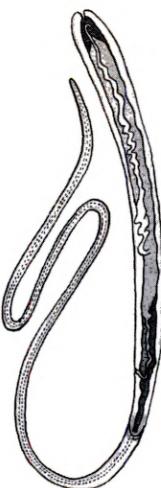
Unfertilized eggs are almost twice as long and somewhat more narrow than fertilized eggs and the outer protective covering and inner shell are thinner. Egg contents consist of large irregular granules and clear non-granular areas. Infertile eggs are encountered in about forty percent of infections and about fifteen percent of all eggs are infertile.

The outer, easily broken lamillated albuminous covering is at times sluffed off and absent. This is particularly true when examinations are preceded by agitation as, for example, concentration techniques employing centrifugation.

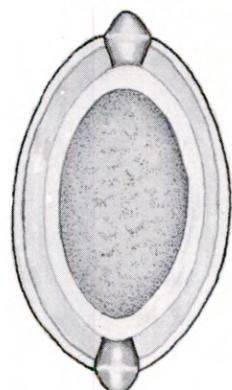
It follows from the above discussion that four types of eggs may be encountered in routine examinations: fertilized corticated, unfertilized corticated, fertilized decorticated, and unfertilized decorticated.

### **Trichuris trichiura (whip-worm)**

*Adult.* Grossly the adults are whip-like in contour, hence the name "whip-worm." Anteriorly more than half the length of the worm consists of a thread-like filament which broadens into the fleshy portion of the body toward the posterior extremity.



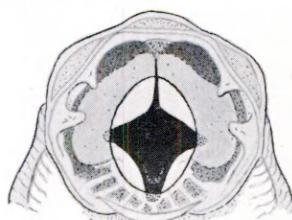
**Adult Female**



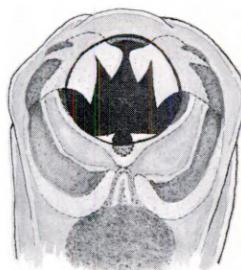
**Ova**

### **Necator americanus Ancylostoma duodenale (hookworms)**

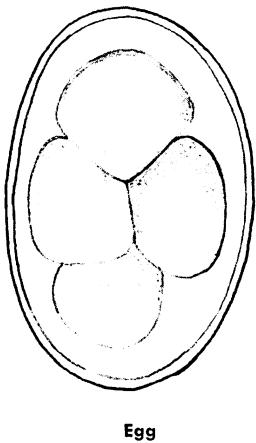
*Adult.* Mature worms are a little less than an inch in length. The two common species which infect man can be differentiated from each other on the basis of the number, type and arrangement of teeth in the buccal cavity, the position of the genital pores in the females, and the number and arrangement of supporting rays and spines in the caudal bursa.



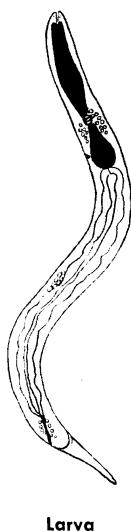
**Head, Necator americanus**



**Head, Ancylostoma duodenale**



Egg



Larva

*Eggs.* The eggs of all species of hookworm of man and lower animals are identical and cannot be differentiated. Hence they are referred to as hookworm ova without reference to species designation. The eggs are ovoidal in shape, glass clear, uncolored, and have smoothly rounded ends. They are approximately one and one-half times as long as wide. In fresh fecal specimens ova will usually contain embryos in the four to eight cell stage of development. In constipated stools or in situations where stool examination is delayed following passage fully embryonated eggs which contain a motile larva may be seen. These eggs may hatch in the stool specimen releasing free larvae which are easily confused with those of *Strongyloides stercoralis*.

### **Strongyloides stercoralis**

*Larvae.* Eggs are not normally passed in the stools of humans harboring this species of roundworm. If on occasion, eggs are eliminated by the host they are morphologically indistinguishable from those of hookworm and would, therefore, be likely designated as such. The eggs usually hatch in the intestine liberating the first stage or rhabditiform larva. As stated above under certain conditions in hookworm infection motile larvae may be encountered in fecal examinations. The larvae of the present species can be differentiated from those of hookworm by the fact that in *Strongyloides* the esophagus is much longer and the larvae are considerably larger.

**Table 6. A Comparative Table of the Two Common Human Hookworms**

Diagnostic Characters	<i>Ancylostoma duodenale</i>	<i>Necator americanus</i>
Common Name-----	Old world hookworm-----	American hookworm—New world hookworm.
Geographical distribution	Europe, Southern Africa, India, the Far East, the Pacific Islands, Northeastern Australia, and a limited portion of the east central coast of South America.	Warmer regions of the entire western hemisphere, the southern half of the continent of Africa, India, the Far East, Northeastern Australia and the islands of the Central Pacific.
Morphology-----	Males and females of almost equal size, measuring 8–13 mm in length. Buccal cavity broadly ovoid, bearing two pair of prominent dorsal teeth and a pair of less prominent ventral cutting plates. The posterior extremity of the female is bluntly pointed while that of the male possesses a broad fan-shaped dorsal bursa within which lie two bristle-like spicules. The latter trail free behind the posterior margin of the bursa. The vulva of the female is posterior to the middle of the body.	Males and females of almost equal size, species smaller and more slender than <i>A. duodenale</i> , measuring 7–11 mm in length. Buccal cavity broadly ovoid, small, armed with a pair of fairly prominent cutting plates on the dorsal aspect and a corresponding pair of markedly less prominent cutting plates on the ventral aspect. Posterior extremity of male roughly similar to the male of <i>A. duodenale</i> , important differences occurring in number and arrangements of dorsal rays and in the appearance of the copulatory spicules. In the present species the latter are fused terminally and end in a backwardly directed barb. The vulva of the female is at about the middle or or anterior to the middle of the body.
Egg production-----	10,000–20,000 eggs daily.	5,000–10,000 eggs daily.
Ova-----	Ovoidal in shape with longitudinal axis only slightly longer than the transverse axis. The single shell is thin and transparent, and in normal stools eggs are usually in the 4–8 celled stage of segmentation when passed.	Similar to <i>A. duodenale</i> .
Cycle in brief-----	Under optimum environmental conditions eggs hatch in less than 24 hours liberating a rhabditiform larva. Within about a week the rhabditiform metamorphoses into the infective filariform larva. Larvae normally enter the host by active penetration of the skin whence they make their way to the lungs via the circulatory system. In the lungs they burrow into the air spaces making their way to the small intestine via the pulmonary and alimentary tracts. Following a period of development in the intestinal submucosa the developing worms attach to the intestinal mucosa and complete the cycle in about 6 weeks.	Similar to <i>A. duodenale</i> .

**Table 7. A Comparative Table of Medically Important Intestinal Nematodes (Other Than Hookworms and *Strongyloides* Sp.)**

Diagnostic Characters	<i>Ascaris lumbricoides</i>	<i>Trichuris trichiura</i>	<i>Enterobius vermicularis</i>
Common name . . .	Giant intestinal roundworm.	Whipworm . . . . .	Pinworm or seatworm.
Geographical distribution.	Cosmopolitan with greatest incidence in moist, warm regions, especially those where human excrement is used as fertilizer.	Cosmopolitan. More common in the warm moist regions of the world, particularly in the tropics.	Cosmopolitan, much more common in children than in adults, infection not dependent upon climatic conditions, but upon personal habits of individuals.
Morphology . . . .	Largest nematode infecting man, mature females sometimes attaining a length of over 40 cm, males considerably smaller. Attachment organ consisting of a dorsal and two ventral lips, the three lips of about equal size forming the apices of a roughly equilateral triangle. Freshly passed worms "fleshy" in color and exhibiting motility. Male readily distinguished from female by marked posterior ventrad curvature.	Males and females of approximately the same size, measuring 30-50 mm in length. Body whip-like anterior portion filamentous and the posterior portion fleshy. Posterior portion of male markedly recurved ventrad, often forming a complete circle. Spicule of male prominent, sheathed. For attachment the anterior thread-like portion of the worm is intertwined into the intestinal submucosa.	Attachment organ consisting of three retractile labia forming a triangle in "in face" view. Females approximately 10 mm in length; males about half as large. Posterior extremity of female marked by attenuation terminating in "pin" point. Male readily distinguished from female by marked posterior ventrad curvature.
Egg production . . .	Capacity of genital tubules around 27 million ova, with estimated egg production of 200,000 daily.	About 2,000 eggs per day . . .	Capacity of genital tubule approximately 20,000 ova. Daily egg production variable.
Ova . . . . .	Variable in size and shape depending upon fertility and presence or absence of albuminous lamellated cortical covering. Fertilized ova broadly ovoid, brown in color, containing circular mass of granular yolk material enclosed in a thick, smooth inner shell, and surrounded by albuminous covering referred to above.	The ova are barrel-shaped and possess transparent polar prominences. They are brown in color and have two shells, an inner thin shell in turn enveloped by an outer thick resistant covering.	Shell moderately flat on one side, broadly oval on the other, clear, hyaline, consisting of an outer rather thin albuminous layer and a thin inner membrane. Ova almost fully embryonated when passed.

**Table 7. A Comparative Table of Medically Important Intestinal Nematodes (Other Than Hookworms and *Strongyloides* Sp.)—Con.**

Diagnostic Characters	<i>Ascaris lumbricoides</i>	<i>Trichuris trichiura</i>	<i>Enterobius vermicularis</i>
Cycle in brief.....	Direct. Embryonation of ova takes place under optimum conditions of temperature, moisture and oxygen within 9 days following passage from host. Under adverse conditions eggs may remain viable for several years. Infection occurs through ingestion of ova containing fully developed rhabditoid larvae. In duodenum the egg hatches, liberating larva which penetrates intestinal wall and by way of the circulatory system reaches the lungs. Following period of development immature worm penetrates out of alveoli and follows air passages to epiglottis from which it follows alimentary tract to small intestine where it reaches maturity in about two months.	Cycle is direct. Embryonation of ova takes place entirely outside of host and requires at least 21 days under optimum environmental conditions. On ingestion larvae are liberated in the small intestine where they undergo partial development within the submucosa. They gradually migrate to the caecum and appendix where they attach and grow to maturity in about three months.	Gravid females migrate to perianal region to deposit ova. Cycle is direct, and complete embryonation takes place within a few hours after ova pass from host. Following ingestion ova pass to duodenum where they hatch and set rhabditoid larva free in lumen. After undergoing some development in duodenum, immature larvae pass to large intestine where they reach maturity and complete the cycle in about three weeks.
Remarks.....	Should be regarded as one of the most dangerous helminth infections of man.	From a therapeutic standpoint this is the most difficult helminth to dislodge from the intestine.	Most common nematode infection of man in the United States.

## PSEUDOPARASITES AND ARTIFACTS

A guide to the identification of objects which may be encountered in routine fecal examinations would be most incomplete if a strong word of caution concerning objects which may fool the unwary or the inexperienced technician was excluded from the discussion. These objects are generally referred to as "pseudoparasites" or "artifacts." Pseudoparasites can be defined as objects which resemble parasites or ova, but are either not parasites at all or are not parasitic in man. Their accurate identification is of much less importance than their separation from objects which are diagnostic

of actual intestinal parasitic infections. Unfortunately, there is no simple yardstick which can be applied as a criterion for determining whether a given object is truly a parasite or whether it is in actuality any one of the myriad of objects which may be present in a stool specimen at the time of examination. Some of the objects more commonly encountered in fecal examinations, which may be confused with true parasites, are illustrated in accompanying figure 3-1.

In distinguishing between objects which are parasites and objects which are not, factors

which must be considered include the conditions under which the specimen may have been collected, the age of the specimen, and technique utilized to prepare the specimen for microscopic observation. It must be emphasized that the error of identification can extend in either direction; that is, objects which are not parasites may be wrongly identified as such, and true parasites may be misidentified as pseudoparasites or artifacts.

Objects which may be incorrectly identified as parasites fall into several broad categories. These include cellular elements which are normally shed into the intestine from various points in the digestive tract, remnants of debris originating from solids ingested by the patient in food or drink, yeasts and fungi which are normal inhabitants of the intestinal tract, and contaminants introduced into the specimen after it has been passed by the patient.

### **Cellular Elements from the Intestinal Tract**

Various cellular elements originating from the intestinal tract may be present and these often closely resemble intestinal protozoan cysts and trophozoites. In certain situations, such as in dysenteric stools or stools from patients suffering from chronic disease of the intestinal tract, these cells may be found in large numbers. Cells commonly encountered are columnar epithelial cells from the gut, squamous cells from the anal mucosa and leukocytes including large endothelial macrophages. Careful observation of structural characteristics of the nuclei, their relative size in relation to the cellular cytoplasm, and the nature of cytoplasmic inclusions are the most diagnostic criteria for distinguishing these cells from protozoans.

In fresh preparations, leukocytes may exhibit pseudopodial activity and motility. Careful observation under varying intensities of light will usually reveal the presence of uniform-sized highly refractile granules easily distinguishable from characteristic protozoal inclusions. In hematoxylin stained preparations, polymorphonuclear leukocytes may exhibit nuclei closely approximating those present in amebic cysts. On close study, it will

generally be seen that these nuclei are larger in relation to the cytoplasm, than in amebic cysts. They also tend to be unequal in size, irregular in outline, and stain more intensely; and they fail to show uniformity of internal morphology corresponding to karyosome bodies and even distribution of inner peripheral chromatin.

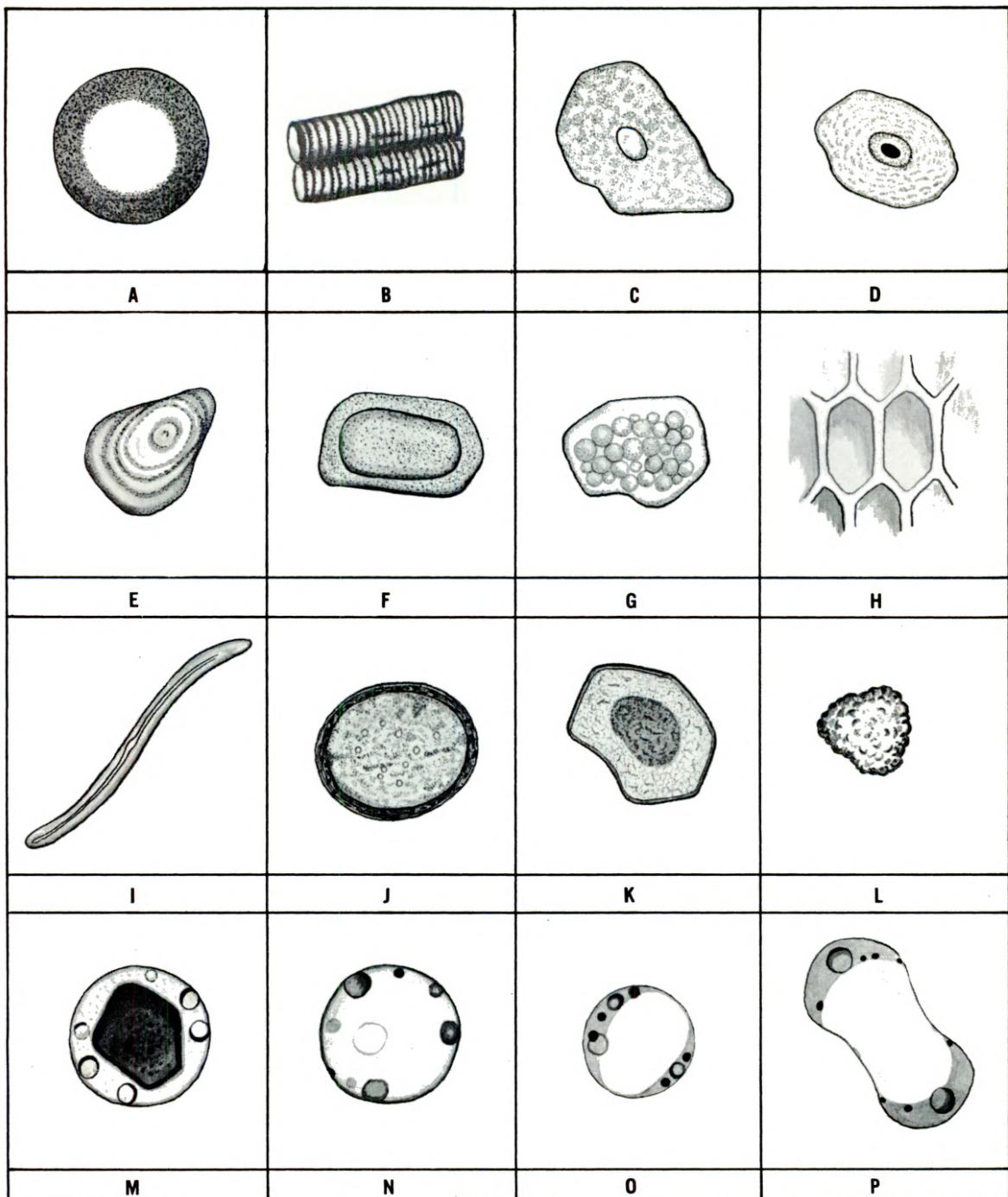
Large endothelial macrophages containing ingested red cells may be mistaken for the trophozoites of *E. histolytica*. In the nucleus the absence of a structure corresponding to the karyosome body, and the presence of fine, rather evenly distributed network of chromatin interspersed with a scattering of relatively large deeply staining particles scattered throughout, usually facilitate recognition. Columnar epithelial and squamous cells are flat and will be noted to lack the dimensional qualities of protozoans as depth of focus is carefully manipulated.

### **Substances Ingested with Food or Drink**

Numerous substances which may be ingested remain sufficiently unaltered in their journey through the intestinal tract that they may be eliminated in various sizes, and in such configurations as to be easily confused with whole parasites, portions thereof, or stages in their development. Among these are cysts of normally free living protozoans, ova or adults of free living nematodes, ova, whole larvae, body parts or whole adult insects, the indigestible cell walls or other portions of plant and animal tissues, and pollen grains.

Free living protozoa that live in fecal material are known as coprozoic species. Cysts of these normally free living forms may pass through the body unchanged. Or, they may be ingested, and after passing through the body, excyst when they are exposed to room temperature. In this manner, both cysts and trophozoites of protozoans which are foreign to the intestine of man may be recovered in fecal examinations.

Soil nematodes are common parasites of the roots of plants. In the case of vegetables which are eaten raw, the possibility of ingestion of the worms or their ova and their ult-



**Figure 3-1. Pseudoparasites and Artifacts Commonly Mistaken for Intestinal Parasites.**

A, oil globule, B, partially digested muscle fiber, C and D, epithelial cells, E thru H, vegetable cells, I, plant hair, J thru L, pollen grains, M thru P, *Blastocystis* sp.

mate recovery in fecal examinations is apparent. While it is known that the ingestion of the ova of certain species of Diptera may give rise to intestinal myiasis, the recovery of either dead or viable dipterous larvae must be carefully interpreted in the light of all possible factors before it can be concluded that larvae present are responsible for this type of parasitic infection. Certain species of oviporous Diptera seek feces upon which to deposit their living young and specimens left unprotected against flies following passage may immediately have maggots deposited upon them. Unprotected fecal specimens may serve as habitats of choice for oviposition by oviporous species. Under optimal conditions of temperature these soon hatch and with resultant maggot infestation of specimens. Since no adult arthropods are known to be associated with parasitism in man, the recovery of portions of, or whole insects is always due to accidental ingestion and subsequent expulsion rather than to parasitism.

Vegetable cells, starch granules, and animal cells from food are of many shapes and sizes and may resemble cysts, ova, or nematodes. Starch granules may be quite spherical, and if undigested, they are composed of successive concentric rings of gray homogeneous material. Potato starch cells often occur in clumps of sack-like, fairly uniform aggregates suggestive of egg packets of *D. caninum*. In iodine stained preparations, undigested starch will stain blue while partially digested starch particles assume a pinkish hue.

The undigested, individual spindle-shaped vesicles of citrus fruit such as oranges and grapefruit are worm-like in outline and may easily be mistaken for adult pinworm. Close examination of the interiors reveals that these sacs are hollow and structureless. Fibrous plant hairs and lint fragments may also be mistaken for nematodes. Ingested pollen grains may be mistaken for helminth ova. Their lack of characteristic yolk granules or developing embryos, and the absence of a clearly delineated shell serve to distinguish them from ova.

## Normal Non-Parasitic Inhabitants of the Intestinal Tract

Perhaps the greatest sources of confusion and error in the identification of objects seen in microscopic examination of feces are yeasts and fungi. These are normal inhabitants of the intestinal tract. *Blastocystis hominis*, a harmless intestinal commensal is the species most easily mistaken for a protozoan cyst. It is spherical in outline and its gradations in size correspond to those of the larger amebae of man. It is characterized by a spherical central mass which is devoid of morphological structures, and the outer wall within which the nuclei are contained is relatively thick and surrounded by a thin capsule. In iodine stained preparations, the central mass does not stain but the thickened outer zone takes on a yellowish-brown color. Within this latter area the nuclei, usually about 3-7 in number, are evenly distributed and very darkly stained. Most of the other species of yeast cells encountered are ovoid in shape and they too, fall within the range of the various protozoan cysts. Yeast cell nuclei are solid, they lack internal morphological structure, and stain blue-black with hematoxylin stains. The best criterion for separating yeasts from parasitic protozoa is the examination of a series of these cells for budding forms.

## Contaminants

The necessity for collecting fecal specimens in clean, covered disposable containers cannot be overemphasized. Glassware in which specimens are processed and mixing devices must be scrupulously clean and free of all extraneous matter. Equally important precautions apply to the care which must be exercised in the selection of diluents to be added to specimens during examination. Failure to exercise these precautions may result in the introduction of all manner of objects which may lead to erroneous embarrassing laboratory errors. Objects which may be negligently introduced include, but are by no means limited to, cysts and trophozoites of free living amoebae, free living ciliates, yeasts, pollen, and fly maggots.

# PART TWO

## EXAMINATION OF BLOOD

### Chapter 4

### MALARIA

---

Malarial parasites are most numerous in the blood and most readily found just before the beginning of a chill. The most satisfactory procedure for the definitive diagnosis of malaria is the use of a thick and thin blood film on the same slide. The thick film serves to concentrate the parasites. Prior to staining,

the thick film is rendered transparent by lysing the red cells and freeing the hemoglobin. Species identification may not be possible from the thick film alone. If this film is positive for malaria parasites, it may be necessary to turn to the thin film for species identification.

### PREPARATION OF BLOOD FILMS

The slide must be chemically clean in order to assure uniform blood films which will adhere to the slide. Cleanse the fingertip or ear lobe with an alcohol sponge, dry with a sterile piece of cotton or gauze, and puncture the skin with a sharp lancet. The thin film is prepared exactly the same way as for routine differential blood count. The film is prepared so as to occupy two-thirds of the length of the slide. The remaining clean end of the slide is used to prepare the thick film. To prepare a thick film, touch the surface of the slide to the drop of blood on the finger or ear lobe, and move the slide in a circular manner until a film about the size of a dime is obtained; this film should be of such thickness that ordinary printing can

barely be read through it. The proper thickness is critical since films which are too thin will contain fewer parasites and those which are too thick tend to crack on drying. The slide may be identified by writing on the thick end of the thin smear with an ordinary pencil. The slides prepared in the above manner are placed in a slide box to protect them from dust and insects until they are stained. The slide is ready for staining after the thick film has dried. It is desirable to stain the slides within a day after preparing them to get maximum staining quality. This is particularly important for the thick film. The latter tends to harden with age and becomes difficult to dehemoglobinize.

### STAINING OF BLOOD FILMS

Of the several stains which can be used for demonstrating malaria parasites, Giemsa's stains the organisms most sharply and deeply and such preparations resist fading for many years. The principal disadvantage of this procedure is that it is considerably more time consuming than the other methods. Wright's stain takes only a few minutes and is, therefore, very good for rapid diagnosis. The JSB Technique is rapid and parasites stand out very

clearly, but preparations stained by this method fade rapidly. Field's stain is rapid and the same stain can be used repeatedly. This technique is not widely used but is excellent for rapid diagnostic work. With this method, if the blood is anemic, staining may have to be prolonged up to 10 seconds and the reticular substance of immature erythrocytes is stained blue and easily confused with malaria organisms. This method was developed and used widely during

World War II and proved very useful where large numbers of blood films were being prepared for microscopic examination. Preparations stained in this manner also fade rapidly and the method is of no value where permanent preparations are desired. All four staining techniques herein described can be utilized for both thick and thin blood smears.

#### **Giemsa's Stain:**

##### *Materials and Equipment:*

Giemsa's Stain (29)

Staining jars

Methyl alcohol

Slides with thick and thin films

Buffered water (pH 7.0) (16)

##### *Technique:*

1. Add 1 part of Giemsa's stain to approximately 50 parts of distilled water. (The optimum dilution will vary with each batch of stain.)
2. Fix the thin film only for 1 minute with methyl alcohol. Do not let alcohol touch the thick film.
3. Allow the slide to dry and then place entire slide in staining solution for 45 minutes.
4. Remove and dip 3 times in buffered water (pH 7.0).
5. Immerse thick portion only in buffered water for 3 minutes.
6. Remove slide and drain dry. Do not blot.

#### **Field's Stain for Thick Films:**

##### *Materials and Equipment:*

Field's stain, solution A and B (25, 26)

Staining jars

Slides with thick blood films

##### *Technique:*

1. Dip thick film for 1-5 seconds in solution A.
2. Rinse by dipping in water until stain ceases to flow from the film.
3. Dip for 1-5 seconds in solution B.
4. Rinse by dipping gently in water for 2-3 seconds.
5. Drain dry.

#### **JSB Stain (Jaswant-Singh-Bhattacherji):**

##### *Materials and Equipment:*

JSB stain solutions A and B (44, 45)

#### **Staining jars**

Slides with thick and thin blood films

##### *Technique:*

1. Thin films.
  - a. Fix smear in methyl alcohol for 1 minute and dry.
  - b. Place in solution A for 30 seconds.
  - c. Wash in water (pH 6.2-6.6)
  - d. Place in solution B for 1 second.
  - e. Wash in same water as step c.
  - f. Replace in solution A for 30 seconds.
  - g. Wash in same water as in step c until the smear has a pink background.
2. Thick films.
  - a. Lake the thick film in water until it has a gray appearance, and dry.
  - b. Place in solution A for 10 seconds.
  - c. Wash in water (pH 6.2-6.6) for 2 seconds.
  - d. Place in solution B for 1 second.
  - e. Wash in same water as in step c until the smear gives a pink background.

#### **Wright's Stain:**

##### *Materials and Equipment:*

Wright's stain (72)

Buffered water (pH 7.0-7.2) (16)

##### *Technique:*

1. Lake the thick film by immersing thick film only in buffered water (pH 7.0-7.2) for 10 minutes.
2. Remove and rinse thick film by dipping in fresh buffered water.
3. Allow film to dry thoroughly. Do not blot.
4. Cover entire slide (thick and thin film) with Wright's stain for 1 minute. (This fixes the film.)
5. Dilute by adding an equal quantity of buffered water and let it stain for 4-6 minutes.
6. Carefully float off metallic scum and rinse by dipping in buffered water and allow to drain dry. Do not blot.

*Note:* For thin films only, start with step 4.

**Table 1. A Comparative Table of the Three Common Species of Human Malaria**

Stages of Development	<i>Plasmodium vivax</i>	<i>Plasmodium malariae</i>	<i>Plasmodium falciparum</i>
Young trophozoite.	Ring form usually with single large chromatin dot. Cytoplasmic circle thin, usually large, pale blue in color.	Ring form with heavy single chromatin dot. Cytoplasmic circle small, thick, and compact.	Ring form with single or double small chromatin dots. Multiple rings in one cell common. Marginal or "applique" rings occasionally occur and are characteristic when present. Rings displaying two chromatin dots common.
Growing trophozoite.	Contour irregular with several finger-like pseudopods. Fine yellowish brown pigment granules in cytoplasm. Chromatin dots single and compact or thread-like, often surrounded by large vacuole.	Cytoplasm compact, frequently in form of a parallel sided or trapezoidal-shaped band across the center of the red cell. Coarse dark brown or black pigment granules often peripherally arranged on margin to trophozoite.	This is usually oldest asexual stage seen in peripheral circulation. It remains in ring form but chromatin and cytoplasm increase in size. Chromatin granules few in number and yellowish in color.
Large trophozoite.	Parasite practically fills enlarged cell. Chromatin in one abundant loose or compact mass. Fine brown hemazion pigment granules abundant.	Parasite fills or almost fills normal sized red blood cell. Pigment granules large, dark, generally peripherally arranged. Cytoplasm dense, dark blue, often in form of a broad band.	Stage seldom seen in peripheral blood. Very small, solid, with one mass of chromatin. Cytoplasm compact, light blue. Very dark pigment scattered throughout cytoplasm or collected in one small dense block.
Presegmenting schizont.	Chromatin divided into two or more irregular masses. Pigment generally clumped into several large particles. Cytoplasm showing various degrees of separation into strands.	Chromatin divided into a few masses. Cytoplasm dense, dark blue in color. Pigment black, evenly distributed throughout parasite.	Rarely seen in peripheral blood. When found parasite is small and pigment is usually clumped in one small dark mass.
Mature schizont or segmenter.	Divided into 12-24 discrete merozoites with oval nuclei surrounded by a similarly shaped mass of cytoplasm. Merozoites arranged either irregularly or in two rings with one or two eccentric clumps or hemozoin granules.	Divided into 6-12 discrete merozoites (usually about 8) generally in a single circle and surrounding a large clump of hemozoin granules. This is called the "rosette" or "daisy" form.	Rarely seen in peripheral blood. Divided into 8-32 very small discrete merozoites. Fills about two-thirds of normal sized red blood cells.

Table 1. A Comparative Table of the Three Common Species of Human Malaria—Continued

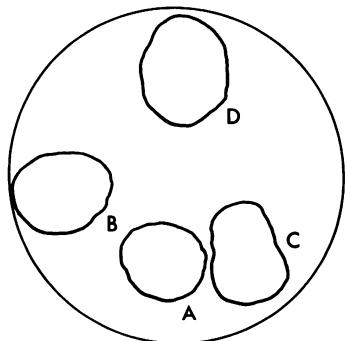
Stages of Development	<i>Plasmodium vivax</i>	<i>Plasmodium malariae</i>	<i>Plasmodium falciparum</i>
Macrogametocyte	Outline circular or ovoid and regular. Cytoplasm homogenous, without vacuoles. Chromatin dot single, small, compact, dark red in color, and eccentric in position. Abundant dark pigment granules scattered throughout cytoplasm.	Outline circular or ovoid and regular. Cytoplasm dense, dark blue, containing abundant coarse dark pigment. Chromatin the same as in <i>P. vivax</i> .	Long, slender, sausage-shaped or crescentic with a concentrated mass of dark pigment near center surrounding a dark red chromatin mass.
Microgametocyte	Usually circular in outline, when fully mature about size of normal red cell. Pigment yellowish-brown abundant throughout cytoplasm. Cytoplasm stains very light blue. Chromatin mass large, diffuse, light red in color at or near center of microgametocyte and often surrounded by a clear vesicular area.	Same as in <i>P. vivax</i> , but smaller. When fully grown usually does not fill normal sized red blood cell.	Cytoplasm generally paler than in macrogametocyte. Parasite broader, shorter, and with more rounded ends than macrogametocyte. Granules of pigment surrounding light staining granules or threads of chromatin throughout central half or more of parasite.
Length of asexual cycle.	48 hours-----	72 hours-----	36-48 hours.
Stages in peripheral blood.	All-----	All-----	Usually ring form trophozoites and gametocytes only. Other stages rarely found except in severe cases.
Time of appearance of gametocytes.	Early, shortly after trophozoites.	Late—Usually 30 days after trophozoites.	Moderately late—about 10 days after trophozoites.
Incubation period in man.	12-17 days, average 14 days	21-35 days, average 28 days	8-14 days, average 12 days.
Period covered by recurrences.	2-3 years-----	7-10 years-----	2-6 months.
Infected red cell	Enlarged, pale, displaying Schuffner's dots.	Normal in size, lacking any characteristic stippling.	Usually normal in size. Maurer's dots sometimes present.
Remarks-----	More developmental stages likely to be observed in one blood film than in any other species.	Parasites compact and hence appear darkly stained.	Parasites usually much more numerous than in other species. Growth of asexual forms other than rings generally takes place in internal organs.

**PLASMODIUM VIVAX**  
**(Tertian Malaria)**

**Color Plates**

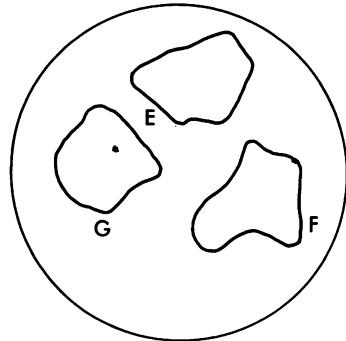
**4-5**

**PLASMODIUM VIVAX (Tertian Malaria) GIEMSA STAIN (x 2000)**



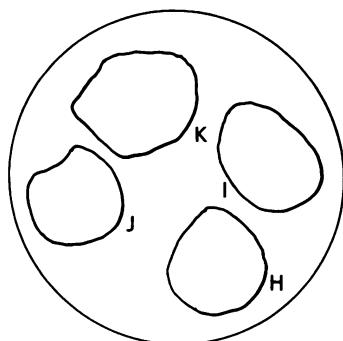
**I. Young Trophozoites**

A and B—Young ring forms.  
C and D—Half-grown trophozoites.



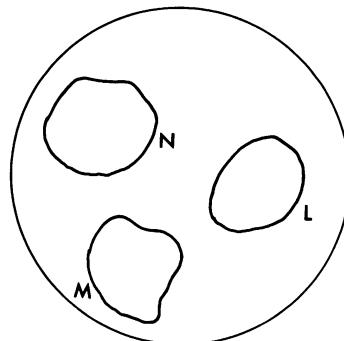
**II. Old Trophozoites**

E, F, and G—Trophozoites with nuclear chromatin ready to subdivide.



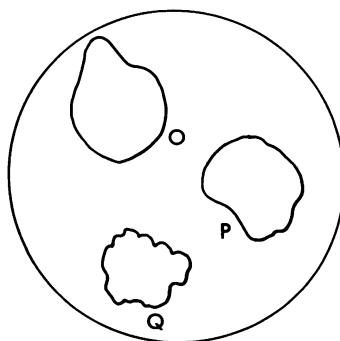
**III. Young Schizonts**

H and I—Young Schizonts showing first division of nuclear chromatin.  
J and K—Older schizonts showing from four to many subdivisions of nuclear chromatin.



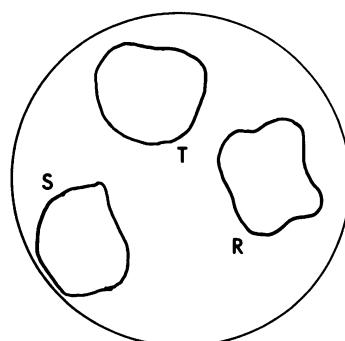
**IV. Half-grown Schizonts**

L, M, and N—Schizonts showing seven, eight, and ten nuclear subdivisions.



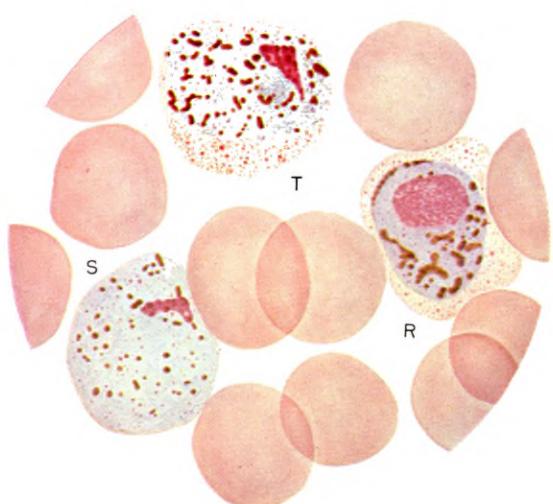
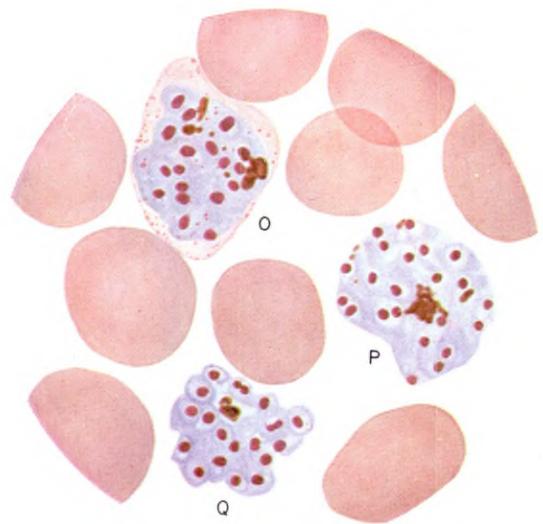
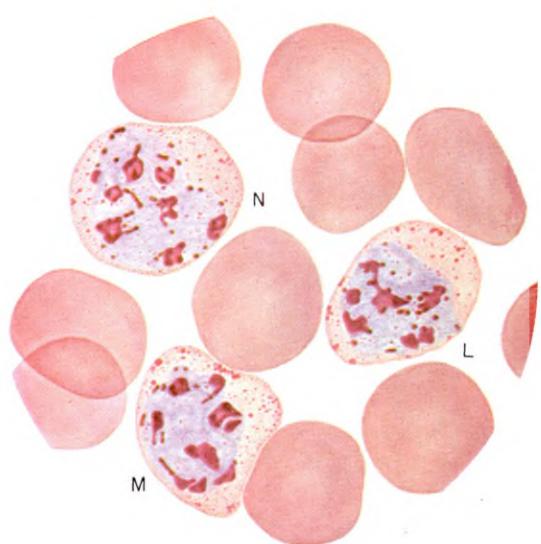
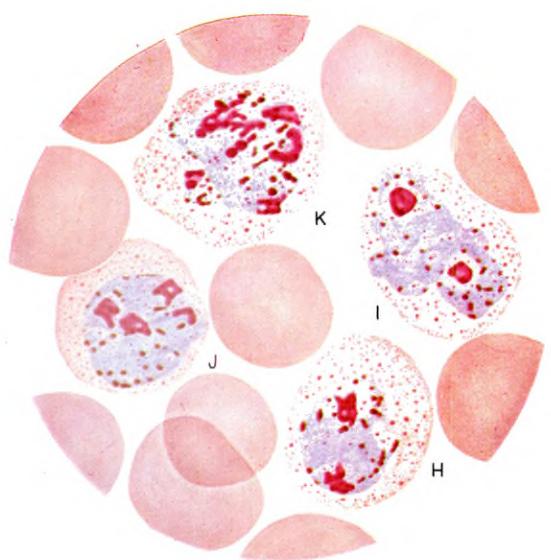
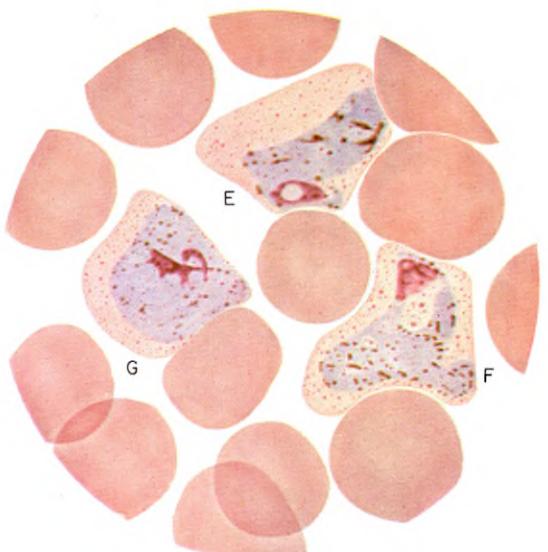
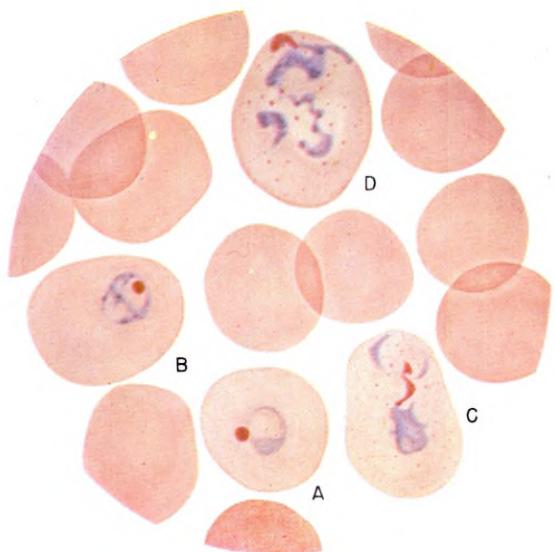
**V. Mature Schizonts**

O, P, and Q—Mature schizonts showing complete subdivision of nuclear chromatin and clumping of malarial pigment.



**VI. Gametocytes or Sexual Forms**

R—Male gametocyte or microgametocyte.  
Note diffuse nuclear chromatin.  
S and T—Female gametocyte or macrogametocyte. Note compact chromatin.

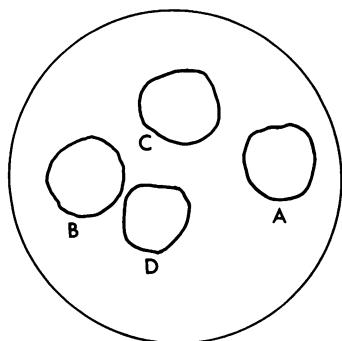




**PLASMODIUM MALARIAE**  
**(Quartan Malaria)**

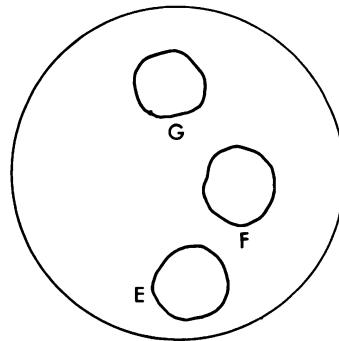
**Color Plates**

## PLASMODIUM MALARIAE (Quartan Malaria) GIEMSA STAIN (x 2000)



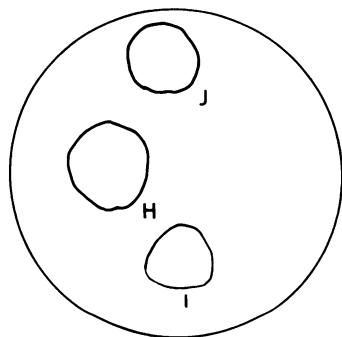
### I. Young Trophozoite

A, B, and C—Progressively older ring forms.  
D—Band trophozoite.



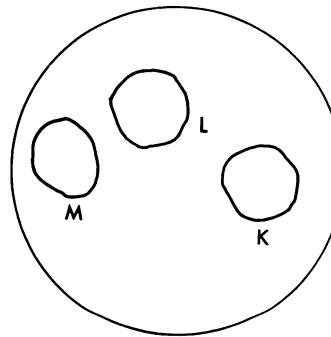
### II. Half-grown Trophozoite

E—Ring form.  
F and G—Mature trophozoites.  
Note amount of pigment; compactness of cytoplasm.



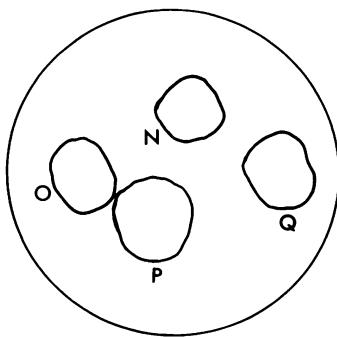
### III. Young Schizonts

H—Band schizont.  
I and J—Three- and five-nucleated schizonts.  
Note large amount of pigment.



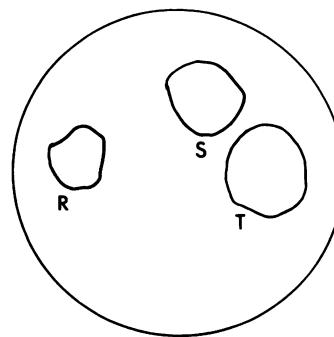
### IV. Half-grown Schizonts

K, L, and M—Four- to six-nucleated schizonts. Note amount of pigment.



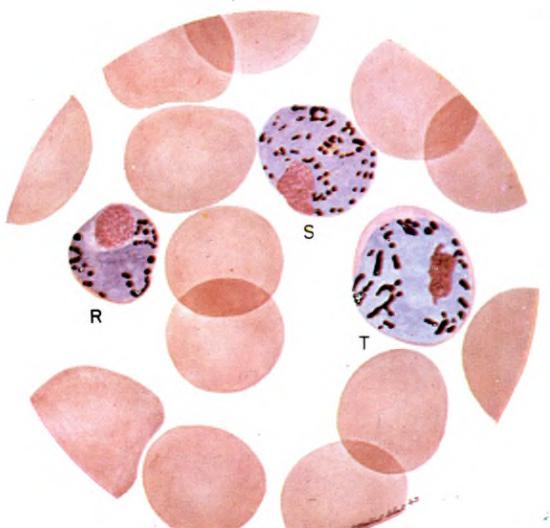
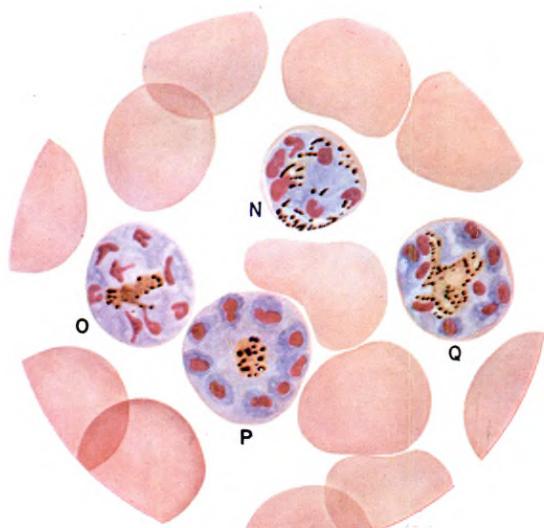
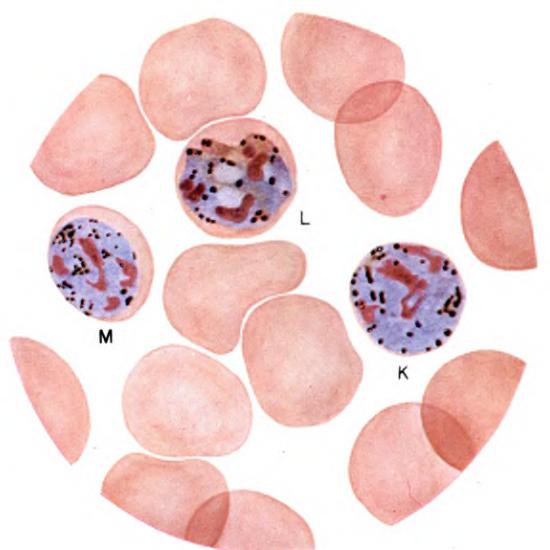
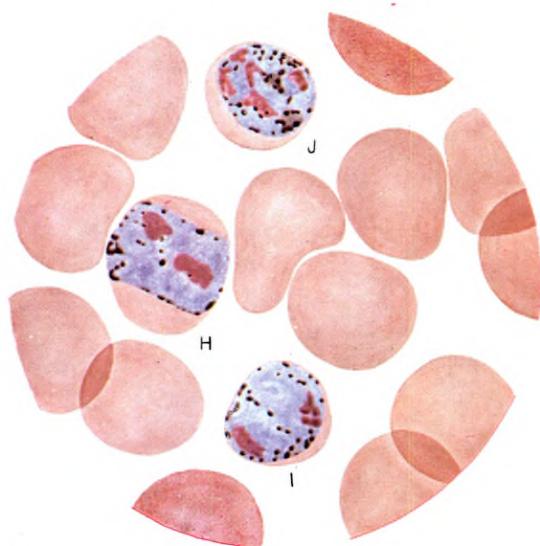
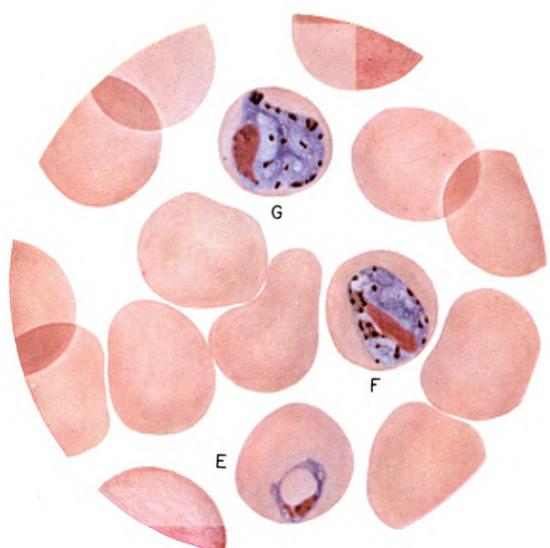
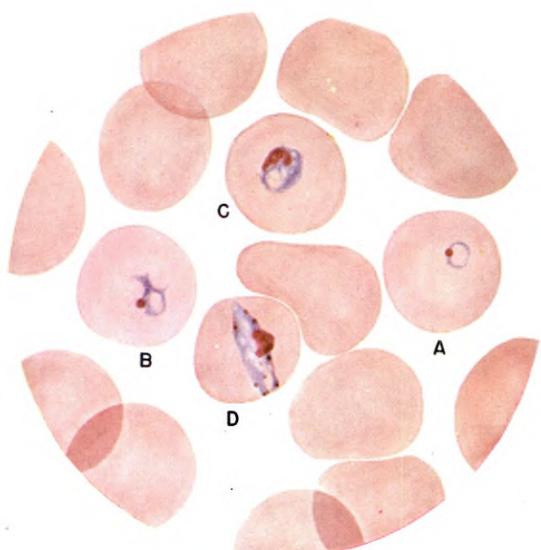
### V. Mature Schizonts

N, O, P, and Q—Eight- or ten-nucleated schizonts ready to segment and release merozoites. Note "daisy" forms.



### VI. Gametocytes or Sexual Forms

R and S—Male gametocytes or microgametocytes.  
T—Female gametocyte or macrogamete.



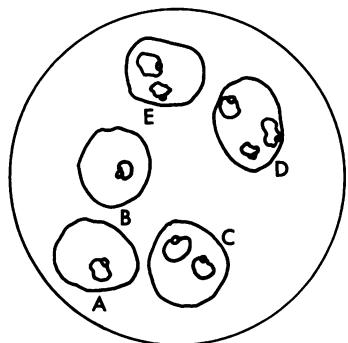


# **PLASMODIUM FALCIPARUM**

**(Estivo-Autumnal Malaria)**

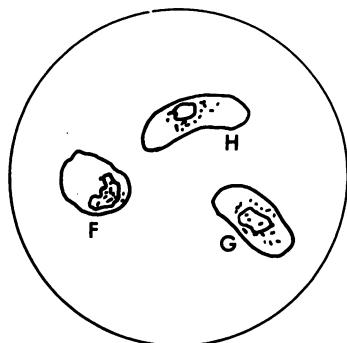
**Color Plates**

**PLASMODIUM FALCIPARUM (Estivo-Autumnal Malaria) GIEMSA STAIN (x 2000)**



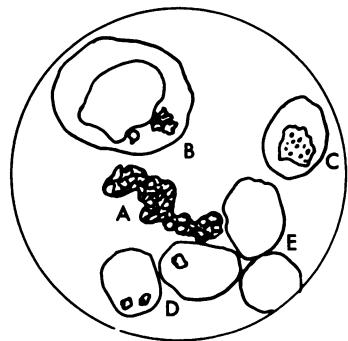
**I. Trophozoites in Peripheral Blood**

- A—Young ring forms.
- B, C, and D—Young trophozoites.
- Note multiple infections of cells and appliqué forms.
- E—These are oldest forms normally found in peripheral blood.



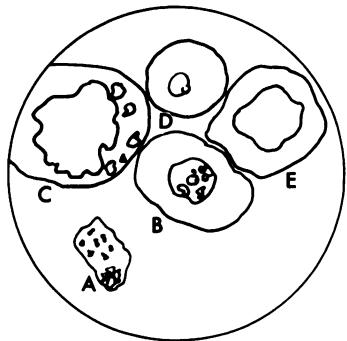
**II. Gametocytes or Sexual Forms in Peripheral Blood**

- F—Gametocyte folded over.
- G—Microgametocyte or male gametocyte.
- Note diffuse chromatin.
- H—Female or macrogametocyte. Note compact chromatin.



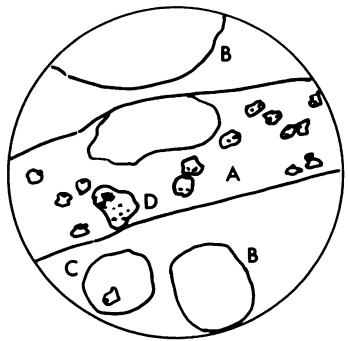
**III. Impression Smear of Spleen**

- A—Free pigment of splenic pulp.
- B—Pigment in macrophage.
- C—Half-grown schizont in cell.
- D—Parasitized red cells.
- E—Lymphocytes.



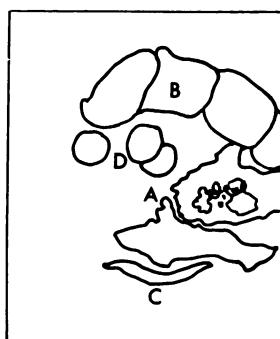
**IV. Impression Smear of Bone Marrow**

- A—Free pigment granules.
- B—Nucleated red cell.
- C—Pigment in macrophage.
- D—Parasitized red cell.
- E—Eosinophile.



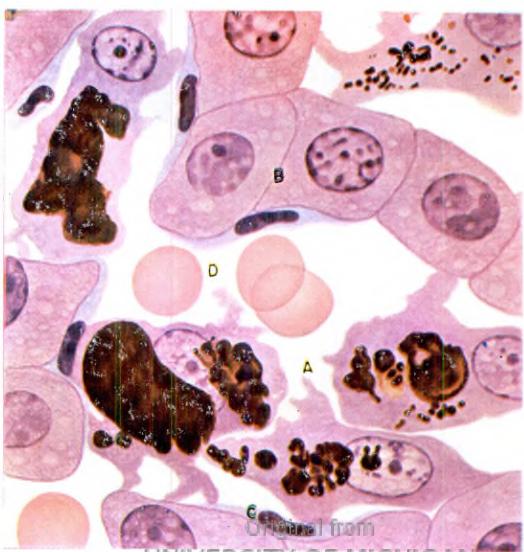
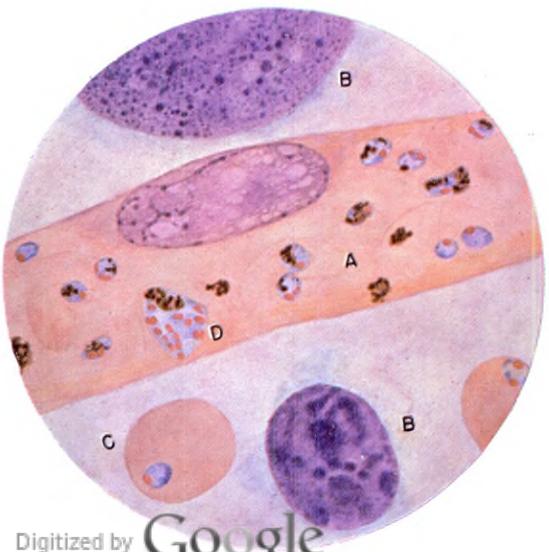
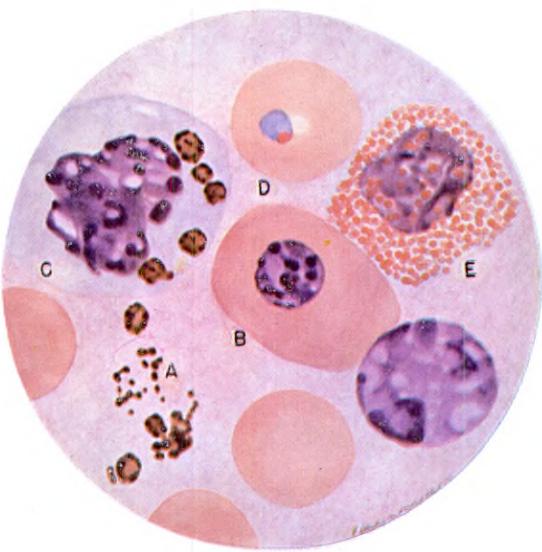
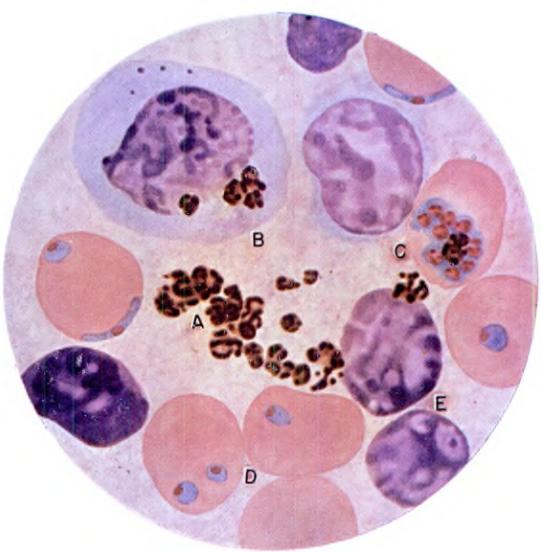
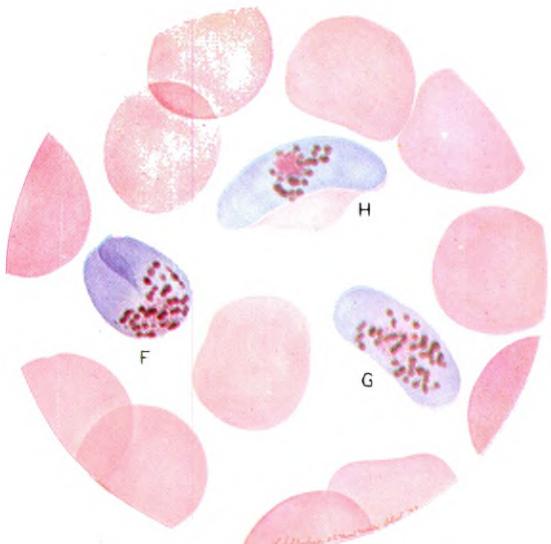
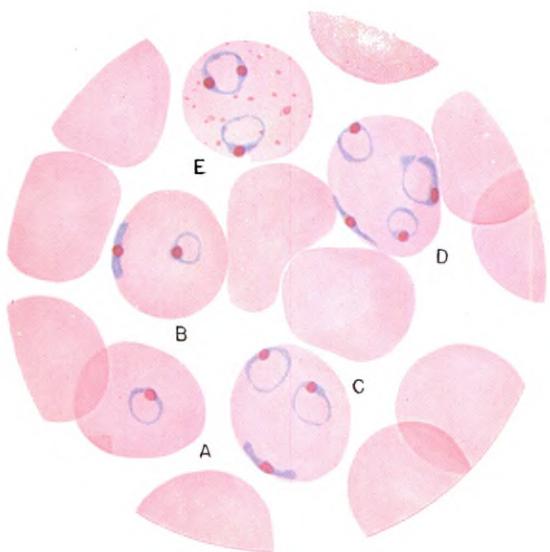
**V. Impression Smear of Brain**

- A—Capillary blocked with parasitized erythrocytes.
- B—Glial cells.
- C—Trophozoite in red cell.
- D—Maturing schizont.



**VI. Section of Liver**

- A—Küpffer cells with pigment.
- B—Hepatic cells.
- C—Endothelial cell.
- D—Free red blood cells.





## Chapter 5

# LEISHMANIASIS AND TRYPANOSOMIASIS

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Laboratory procedures for diagnosis of *Leishmania donovani* include culture of material taken from spleen, liver, bone marrow, lymph node or peripheral blood. Any of the above listed materials can also be stained with Giemsa's or Wright's stain and examined directly for Leishman-Donovan bodies and these materials can also be passed intraperitoneally into hamsters. To inoculate cultures, introduce aseptically amounts up to 1 ml of blood, bone marrow, or macerated tissue from spleen, lymph node, or liver by placing it in the fluid overlay. Incubate 3-14 days at 22° C.-25° C., checking daily after the third day for the characteristic leptomonad forms.

Laboratory procedures for diagnosis of infections with *Leishmania tropica* and *Leishmania brasiliensis* include staining and direct examination of exudate taken from the indurated margins of skin ulcers and the culture of such material on suitable media. Inoculum in amounts up to 1 ml is introduced in the fluid overlay and cultures are incubated at 22° C.-25° C. for 3-14 days with daily checks for characteristic leptomonad forms after the third day.

Diagnostic methods which may be employed for the detection of infections with *Trypanosoma gambiense* and *Trypanosoma rhodesiense* vary with the stage of disease. In the earlier febrile stage the organisms are most likely to be recovered from the blood or macerated tissue from an enlarged lymph node. Methods that may be used are direct examination of stained or unstained smears, inoculation into rats or mice, and culture on suitable media. Cultivation of these two species is difficult, however. In the advanced stage of these diseases direct examination of spinal fluid and culture or animal inoculation of spinal fluid may be per-

formed. In any of the various suggested laboratory procedures recovery of organisms may be facilitated by concentration techniques employing centrifugation. Ten ml quantities of blood or possibly lesser quantities of lymph gland juice are centrifuged at 900-1,000 rpm for 3 minutes. The supernate is pipetted off and centrifuged at 1,800-2,000 rpm for 20 minutes. Sediment can then be examined either stained or unstained and it can also be passed into experimental animals or culture. To concentrate trypanosomes in spinal fluid, centrifuge 5 ml of fluid as 1,500-2,000 rpm for 20 minutes. Again the sediment is examined either stained or unstained. The sediment can also be passed into culture or experimental animals.

The demonstration of *Trypanosoma cruzi* in the blood is difficult since the organisms occur in the peripheral blood only during the acute stage and during febrile periods in the chronic stage of the disease. Negative findings in direct examination of either fresh or stained blood films should be followed by inoculation of cultures. To inoculate cultures place ½-2 ml quantities of blood into the fluid overlay of suitable culture media. Animal inoculation should also be performed in mice, guinea pigs, or rats (free from *T. lewisi*). Inoculate experimental animals with ½-2 ml quantities of blood depending upon the size of the animal utilized. In both culture and experimental animals *T. cruzi* is much more readily recovered than the organisms of African sleeping sickness. Both cultures and animals should be checked periodically from the second through the fourth week following inoculation. Trypanosomes also revert to leptomonad forms in culture.

## PREPARATION OF MEDIA FOR THE CULTURE OF TRYPANOSOMES AND LEISHMANIA

### Diamond's SNB 9 Medium:

#### Materials and Equipment:

Water bath	
Test tubes . . . . .	15.00 ml
Penicillin-Streptomycin so- lution	
Cotton, absorbent	
Neopeptone (Difco) . . . .	18.00 gm
Sodium chloride . . . . .	4.95 gm
Distilled water . . . . .	1000.00 ml
Agar . . . . .	18.00 gm
Defibrinated rabbit's blood <sup>1</sup>	0.5-1.25 ml

<sup>1</sup> Quantity required for each tube of medium.

#### Technique:

1. To prepare base stock add reagents in the order listed and dissolve thoroughly by supplying moderate heat if necessary by means of water bath. Tube medium in 5 ml quantities in 15 ml test tubes, plug tubes with cotton and autoclave for 20 minutes at 15 pounds pressure.
2. To prepare the medium melt it by bringing it up to a temperature of 45°-50° C. To each tube add  $\frac{1}{2}$  ml of defibrinated rabbit's blood, mix thoroughly, and cool in slants. The medium can be enriched by adding  $1\frac{1}{4}$  ml defibrinated rabbit's blood to each tube for initial isolation of flagellates. The addition of 1,000 units of penicillin and 1,000 units of streptomycin to each tube is also recommended for initial isolation of flagellates.
3. To prepare overlay add neopeptone and sodium chloride to distilled water in quantities indicated in the base stock formula above and sterilize in the autoclave. When slants have hardened add  $\frac{1}{2}$  ml of overlay to each tube.

### Kelser's Medium for the Cultivation of Trypa- nosoma Cruzi:

#### Materials and Equipment:

Flask, Erlenmeyer . . . . .	1,000 ml
Water bath	

Cotton, absorbent	
Paper, litmus	
Flask, Erlenmeyer . . . . .	250 ml
Pipette . . . . .	10 ml
Test tubes . . . . .	15 ml
Paraffin	
Bacto-Beef . . . . .	25.0 gm
Bacto-Peptone . . . . .	12.5 gm
Sodium chloride . . . . .	3.5 gm
Bacto-Agar (granular) . . . .	5.0 gm
Dextrose solution, 1 per- cent . . . . .	10.0 ml
Distilled water . . . . .	500.0 ml
Sodium hydroxide solu- tion <sup>2</sup> . . . . .	1 N
Defibrinated rabbit or guinea pig blood <sup>3</sup> . . . . .	10.0 ml

<sup>2</sup> To prepare 1 N sodium hydroxide, place 4 gm sodium hydroxide in a 100 ml graduate and q.s. to 100 ml with distilled water.

<sup>3</sup> Quantity required for each 100 ml of stock medium.

#### Technique:

1. To prepare base stock place 500 ml of distilled water in a 1,000 ml Erlenmeyer flask and add the Bacto-Beef.

Heat this mixture in a water bath at 55° C. for 1 hour. Following heating add the Bacto-Peptone and sodium chloride and bring the water bath to boiling. At this temperature heat for 5 minutes, shaking the flask several times to bring all ingredients into solution. Filter the broth through absorbent cotton 2 times. Using 1 N sodium hydroxide solution and litmus make the broth neutral. Following precise neutralization of the medium again place it in a hot water bath (boiling) and add the Bacto-Agar. When the Bacto-Agar is thoroughly dissolved dispense the medium in 100 ml amounts in Erlenmeyer flasks and sterilize in the autoclave at 12 pounds pressure for 30 minutes. The base stock can then be stored in the refrigerator until ready for use.

2. To prepare the medium for use place an Erlenmeyer flask containing 100 ml of the stock in a hot water bath

until the agar is melted. Lower the temperature of the medium to 50° C. and add aseptically the 10 ml of 1 percent dextrose solution and 10 ml of defibrinated blood. With a sterile 10 ml pipette transfer 5 ml quantities of the medium to 15 ml test tubes. Plug the tubes with sterile cotton, slant, and let stand until hardened. Paraffin the cotton plugs and place immediately in the refrigerator overnight to assure maximum water of condensation. To test for sterility incubate at 37° C. overnight. Store in refrigerator until ready for use.

#### NNN Medium for Leishmania:

##### Materials and Equipment:

Flask, Erlenmeyer .....	1,000 ml
Flasks, Erlenmeyer .....	250 ml
Paper, litmus	
Pipette .....	5 ml
Test tubes .....	15 ml
Bacto-Agar .....	14 gm
Sodium chloride .....	6 gm
Distilled water .....	900 ml
Sodium hydroxide solution <sup>2</sup> .....	1 N
Rabbit or guinea pig defibrinated blood <sup>4</sup> .....	10 ml

<sup>2</sup> To prepare 1 N sodium hydroxide, place 4 gm sodium hydroxide in a 100 ml graduate and q.s. to 100 ml with distilled water.

<sup>4</sup> Quantity required for each 150 ml of stock medium.

##### Technique:

1. To prepare base stock mix the distilled water, Bacto-Agar, and sodium chloride in an Erlenmeyer flask. Bring to a boil and then neutralize with 1 N sodium hydroxide using litmus paper as an indicator. Distribute the stock medium in suitable containers in 150 ml quantities. Sterilize at 12 pounds pressure for 15 minutes and store in refrigerator until ready for use. The stock medium will keep for several months at refrigeration temperature.
2. When ready to add the defibrinated blood heat a flask of base stock to

boiling, then cool to 50° C. Add 10 ml of defibrinated blood aseptically, mix thoroughly and pipette 5 ml quantities of medium into 15 ml test tubes. Slant and when the medium has hardened place overnight in the refrigerator to enhance formation of water of condensation. Incubate at 37° C. overnight to test for sterility before use.

#### Offutt's Medium for Cultivation of *T. Cruzi*, *T. Lewisi*, and the Leishmanias:

##### Materials and Equipment:

Flask, Erlenmeyer .....	1,000 ml
Water bath	
Flasks, Erlenmeyer .....	250 ml
Test tubes .....	15 ml
Cotton, absorbent	
Paraffin	
Bacto Blood-Agar Base...	40 gm
Distilled water .....	1,000 ml
Defibrinated rabbit's blood <sup>5</sup> .....	10-20 ml
Sterile buffered saline or sterile Locke's solution <sup>6</sup> .	0.5-1 ml

<sup>5</sup> Quantity required for each 200 ml of stock medium.

<sup>6</sup> Quantity required for each tube of medium.

##### Technique:

1. To prepare base stock add the Bacto Blood-Agar Base to the distilled water in an Erlenmeyer flask, soak for 5 minutes, mix thoroughly and heat in a water bath until the agar is thoroughly dissolved. Distribute base stock in 200 ml amounts, and sterilize in the autoclave at 15 pounds pressure for 20 minutes. The stock is stored in the refrigerator until ready for use.
2. When ready to add the defibrinated blood heat one flask of the base stock to boiling, then cool to 45°-50° C. Add aseptically 10-20 ml of defibrinated rabbit's blood, mix thoroughly and tube in 4-5 ml amounts in sterile 15 ml test tubes. Allow the medium to solidify in slants. A fairly short slant with deep butt is preferred. Add  $\frac{1}{2}$ -1 ml

of sterile Locke's solution or sterile buffered saline to each tube. Test for sterility by incubating for 24 hours at 37° C. Paraffin cotton plugs to prevent evaporation of overlay.

#### **Senekjie's Modified Medium for *Trypanosoma cruzi* and *Leishmania*:**

##### *Materials and Equipment:*

Flask, Erlenmeyer.....	1,000 ml
Water bath	
Funnel	
Paper, filter	
Flasks, Erlenmeyer .....	250 ml
Test tubes.....	15 ml
Pipette.....	10 ml
Cotton, absorbent	
Paraffin	
Bacto-Beef.....	50 gm
Distilled water.....	1,000 ml
Neopeptone.....	20 gm
Sodium chloride.....	5 gm
Noble special agar <sup>7</sup> .....	20 gm
Defibrinated rabbit's blood <sup>8</sup> .....	10 ml
Sterile Locke's solution <sup>9</sup> ..	2 ml

<sup>7</sup> Noble special agar is a highly purified agar available under corresponding trade name.

<sup>8</sup> Quantity required for each 90 ml of stock medium.

<sup>9</sup> Quantity required for each tube of medium.

##### *Technique:*

1. To prepare base stock dissolve the Bacto-Beef in the distilled water and heat to 50° C. for 1 hour in a hot water bath. Raise the temperature to 80° C. for 5 minutes to coagulate proteins, then filter through filter paper. Add the neopeptone, sodium chloride and agar and adjust pH to 7.2-7.4. Autoclave at 15 pounds pressure for 20 minutes. Distribute base stock in Erlenmeyer flasks in 90 ml quantities and store in the refrigerator.
2. When ready to add the defibrinated blood place an Erlenmeyer flask containing 90 ml of the base stock in a hot water bath and heat until agar is melted. Lower the temperature to 50° C. and add aseptically 10 ml of defibrinated rabbit's blood. With a sterile 10 ml pipette transfer 5 ml quantities of the medium to 15 ml test tubes. Plug the tubes with sterile cotton, slant, and let stand until hardened. Overlay with 2 ml of sterile Locke's solution. Paraffin the cotton plugs and place in incubator overnight at 37° C. to test for sterility. Store in refrigerator until ready for use.

**Table 1. A Comparative Table of the Medically Important Trypanosomes**

Characteristics	<i>Trypanosoma gambiense</i>	<i>Trypanosoma rhodesiense</i>	<i>Trypanosoma cruzi</i>
Common name of disease produced.	African sleeping sickness or more specifically Gambian sleeping sickness.	African sleeping sickness or more specifically Rhodesian sleeping sickness.	Chagas' disease.
Geographical distribution.	Wide area in tropical West Africa from Senegal to Loanda extending inland along the large rivers, particularly the Congo and Niger. Newer areas include Southern Sudan, Uganda, and the Great Lakes region.	Eastern Africa from Kenya to southern Rhodesia and northern Mozambique, and inland across Tanganyika to Uganda and eastern Congo.	Extends from pampas of Argentina to deserts and canyons of southern California, Arizona, and Texas. Few cases occur in Mexico and Central America, common in many parts of Venezuela, French Guiana, Brazil, Bolivia, Chile, Paraguay, and Uruguay.

**Table 1. A Comparative Table of the Medically Important Trypanosomes—Continued**

Characteristics	<i>Trypanosoma gambiense</i>	<i>Trypanosoma rhodesiense</i>	<i>Trypanosoma cruzi</i>
Vector and mode of transmission.	Flies of the genus <i>Glossina</i> . "Metacyclic" infective trypanosomes occur in the salivary glands. Transmitted by bite. Mechanical transmission is possible up to 30 minutes after fly feeds on infected individual.	Flies of the genus <i>Glossina</i> . "Metacyclic" trypanosomes occur in the salivary glands. Transmitted by bite. Mechanical transmission possible as with <i>T. gambiense</i> .	Bugs of the genus <i>Triatoma</i> . "Metacyclic" infective forms occur in the anal region. Transmitted through contamination of broken skin by insect droppings.
Localization of the parasites within the vertebrate host.	Scarce in blood, usually more abundant in juice of enlarged lymph glands. Also occur in spleen which is often enlarged. In late stage of the disease they may be found in the cerebrospinal fluid and within tissues of central nervous system. Invasion of the central nervous system takes place later in the course of the disease than in infections with <i>T. rhodesiense</i> .	Scarce in blood, usually more abundant in juice of enlarged lymph glands. Also occur in spleen which is often enlarged. In late stage of the disease they may be found in the cerebrospinal fluid and within tissues of central nervous system. This is usual course of the disease in the case of infections with <i>T. rhodesiense</i> .	Organism invades cells of the heart, voluntary muscles, various glands, and late in the course of the disease, the central nervous system. Organisms usually scarce in the circulating blood.
Clinical symptoms of the disease.	Course irregular, generally milder than Rhodesian type of the disease and of much longer duration. Initial symptoms include fever and headache recurring irregularly followed by weakness, enlarged glands, edema and muscular tenderness. In the late stages there is physical depression, stupor, unconsciousness, coma, and death.	Organism is apparently more virulent and death frequently occurs within 3 or 4 months following infection. Initial symptoms include fever and headache recurring irregularly followed by weakness, enlarged glands, edema, and muscular tenderness. In the late stages there is physical depression, stupor, unconsciousness, coma and death.	Initial stage swelling of eyelids, and conjunctiva, and edema of other parts of face, usually one side only. Lymph glands of neck swell; there may be severe headache, and prostration with more or less continuous fever. In the chronic stage there is extensive hard edema, inflamed glands and enlargement of liver and spleen. Late stage is characterized by extreme weakness, convulsions, paralysis and disturbance of heart function. Intercurrent diseases common and frequently immediate cause of death.
Laboratory diagnosis.	Fresh specimen, thin- or thick-stained smear, hanging drop, centrifugation, xenodiagnosis, animal inoculation, culture, spinal fluid examination, or biopsy of lymphatics.	Same as for <i>T. gambiense</i> , the method of choice usually depending upon the stage of the disease.	Same as for <i>T. gambiense</i> , the method of choice generally depending upon the stage of the disease.

**Table 2. A Comparative Table of the Medically Important Leishmania**

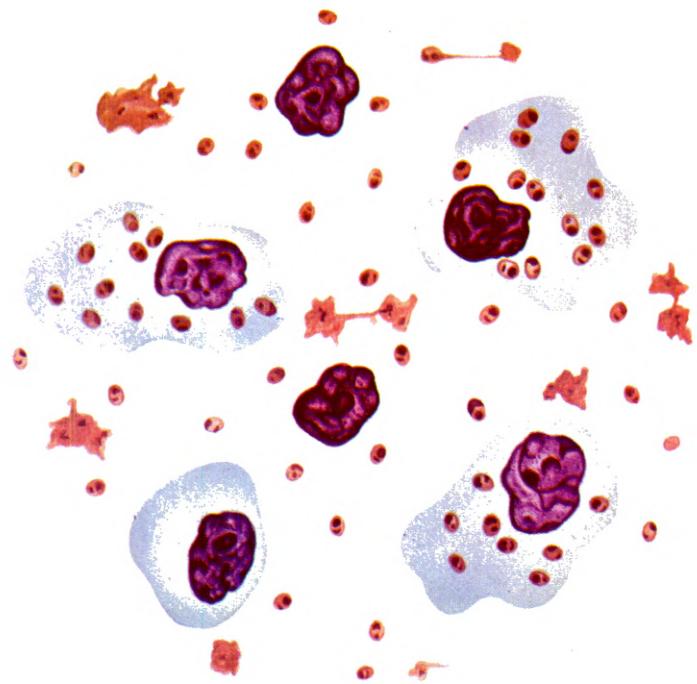
**GENERAL CHARACTERISTICS OF THE GENUS LEISHMANIA:** 1. Rounded forms containing a nucleus and kinetoplast but lacking a flagellum. 2. Any of the other three types of "hemoflagellates" (Leptomonas, Crithidia, and Trypanosoma) may assume this form and may also be developed out of it. 3. In addition to the Trypanosoma this is the only other form of "hemoflagellate" which may be found in the circulating blood of man. 4. The three presently recognized types of human Leishmaniasis are all caused by morphologically indistinguishable parasites, separable on basis of clinical manifestations and geographical distribution. 5. Dogs constitute the principal reservoir animals of Kala-azar and Oriental sore.

Characteristics	<i>Leishmania donovani</i>	<i>Leishmania tropica</i>	<i>Leishmania braziliensis</i>
Common name of disease produced.	Kala-azar, or visceral leishmaniasis.	Oriental sore, or cutaneous leishmaniasis.	Espundia, or South American mucocutaneous leishmaniasis.
Geographical distribution.	In the old world it is common in India, North China, and the Sudan. Around the Mediterranean and in western and middle Asia infantile type of disease occurs. In recent years it has been discovered that the disease is widespread in South America (parts of Brazil, Argentina, Paraguay, Bolivia, and Venezuela).	Common in tropical cities of eastern Mediterranean, usually in children under 3 years. In these regions dogs are frequently infected and are an important reservoir of the disease.	Form of cutaneous leishmaniasis which is frequently followed by involvement of mucous membranes of nose and pharynx. Occurs over large area of tropical America from Yucatan in Mexico through Central America into northern Argentina.
Vector and mode of transmission.	Flies of the genus <i>Phlebotomus</i> are proven natural vectors although other insects may transmit the disease. Transfer of fomites from infected human or reservoir animals can transmit the disease. Transmission generally by bite of the fly.	Generally by flies of the genus <i>Phlebotomus</i> although direct mechanical transmission is definitely possible. Most common vector is <i>Phlebotomus papatasi</i> , a sandfly found wherever this disease occurs. Transmission generally by bite of fly.	Highly probable that <i>Phlebotomus</i> flies are generally responsible. This cycle has not been thoroughly worked out as has that of the other two forms, but naturally infected <i>Phlebotomus</i> have been found in areas where the disease is endemic.
Localization of the parasites.	Leishman-Donovan bodies are widely distributed within the body but special habitat seems to be large endothelial cells of blood vessels and lymphatics. Abundant in spleen, liver, bone marrow, and sometimes found in circulating blood within the monocytes.	Parasites are found in the dermal tissues of infected sores where increased numbers of large monocytes and other reticuloendothelial cells are literally packed with them.	Initially parasites are localized in cutaneous sores as in the case of <i>L. tropica</i> . As disease advances they localize in mucous membranes of nose and pharynx.

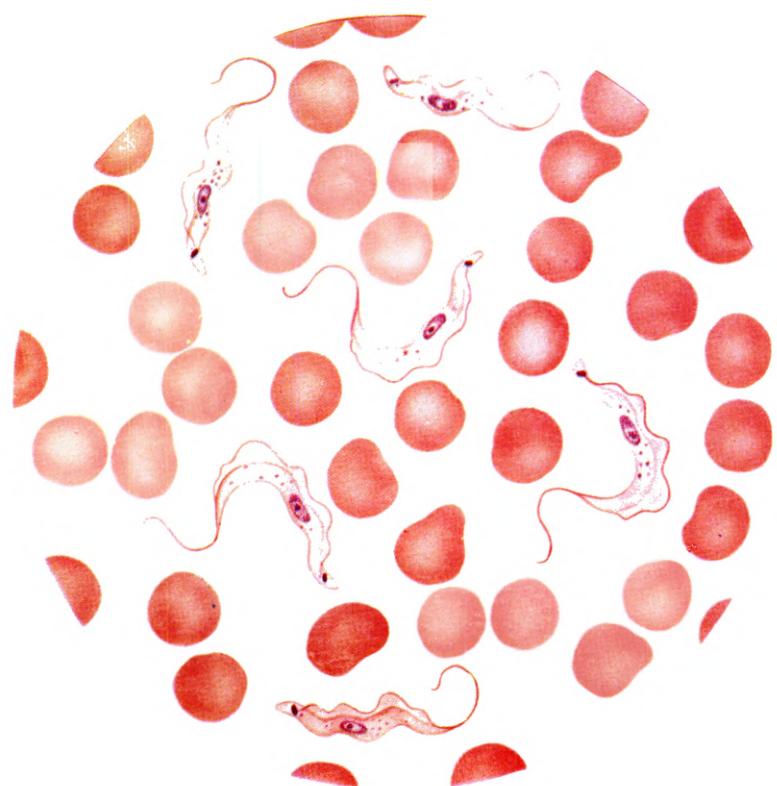
**Table 2. A Comparative Table of the Medically Important Leishmania—Continued**

Characteristics	<i>Leishmania donovani</i>	<i>Leishmania tropica</i>	<i>Leishmania braziliensis</i>
Common name of disease produced.	Kala-azar, or visceral leishmaniasis	Oriental sore, or cutaneous leishmaniasis.	Espundia, or South American mucocutaneous leishmaniasis.
Clinical symptoms of the disease.	Onset is accompanied by irregular fever, rheumatic aches, anemia, and progressive emaciation. Macrophages increase tremendously in number and size so that involved organs including liver and spleen increase markedly in size also. Leukocytes decrease in number, general edema of skin is present. Untreated persons die within a few weeks to several years.	Begins as small red papule which enlarges to an inch or more in diameter. In later stages these develop into unsightly ulcerating sores. Sores persist for a few months to a year or more. They finally heal spontaneously and generalized invasion of the body does not occur.	Typical cases begin initially in a manner similar to Oriental sore except that there is greater tendency for the sores to spread over extensive areas and for numerous sores to appear. In later stages of the disease secondary ulcerations occur in nasal cavities, mouth and pharynx, these generally developing several months to several years after cutaneous lesions appear. Mucocutaneous involvement results in clogging of nostrils, fetid breath, affection of organs of smell, hearing and sometimes sight. In advanced stages the nasal septum and surfaces of the palate are destroyed. Death always occurs in advanced untreated cases, but patients may live several years.
Laboratory diagnosis	Liver or spleen puncture, biopsy of lymph glands, with culture of material recovered, culture of blood, and direct examination of stained blood smears.	Animal inoculation, culture and direct examination of exudates from sores (latter procedure generally confirms positive cases).	Intradermal tests are useful. Direct examinations of exudates from involved areas, culture and animal inoculation are practical laboratory procedures.





Leishmaniasis in macerated hamster spleen. Note intracellular and extra-cellular Leishman-Donovan bodies.



Trypanosoma lewisi in blood smear from an infected rat.



## Chapter 6

# FILARIASIS

Definitive diagnosis of filarial infections is dependent chiefly upon the demonstration of the embryonic stage of the worm in the peripheral blood. With the exception of *Onchocerca volvulus*, all filarial infections will at some stage in the course of the infection display microfilaria in the circulating blood. The time of day when blood is taken from the patient must take into account the type of periodicity

characteristic of the species of filarial worm present in the area where the patient may have acquired the infection. *Wuchereria bancrofti* is usually nocturnal and *Wuchereria malayi* is also nocturnal though less absolutely so than *W. bancrofti*. *Acanthocheilonema perstans* and *Mansonella ozzardi* are non-periodic but tend to be more numerous nocturnally while *Loa loa* displays a diurnal periodicity.

### LABORATORY PROCEDURES

Microfilariae may be demonstrated by several techniques. These include the examination of fresh blood from motile organisms, examination of stained thick blood films and various concentration techniques. The latter should be resorted to when the more direct methods of examination are negative since they enhance the chances of finding the worms in low grade infections.

#### Examination of Fresh Blood

##### Materials and Equipment:

Blood lancet  
Gauze  
Pipette, red-cell  
Slide  
Coverslip

##### Technique:

1. Standard methods of finger puncture are used to obtain the blood specimen. The finger is first thoroughly cleansed using lintless material such as a good grade of gauze. Strands of lint are easily mistaken for microfilariae by the inexperienced observer.
2. With a blood pipette draw up the blood and discharge a large drop on a slide being careful not to form

bubbles in the drop. To prevent formation of bubbles, do not discharge the entire quantity of blood from the pipette onto the slide.

3. Apply a coverslip and search the entire preparation for motile worms under low power.
4. An alternative method is to touch a clean coverslip to the drop of blood on the finger and quickly withdraw it, carrying away a large drop of blood.
5. The coverslip is then placed on a slide, blood side down, and examined in the aforementioned manner.
6. A third method which is particularly useful for demonstrating the motile worms is the hanging drop method. Blood is placed on a coverslip in the manner described above for the coverslip method. The coverslip is then carefully inverted onto a concavity slide with the drop of blood suspended in the well on the slide. The lashing motion of the motile worms moves blood cells about more violently than when a cover-

slip is appressed upon them, thus facilitating their detection.

**Note:** A very useful modification which can be used with any of the above three techniques is to rupture the red cells by adding an equal amount of distilled water to the drop of blood. Allow to stand for a few minutes and the blood cells will rupture leaving the microfilariae actively swimming about in a clear field.

### **Examination of Stained Thick Blood Films**

This technique offers various advantages. A relatively large sample of blood is obtained. It serves as a convenient method for forwarding specimens to a central laboratory for examination or for holding the specimen for examination at a later time. It is an especially convenient method for performing field surveys on large numbers of individuals in endemic regions. Staining reveals structures which make species differentiation relatively simple while microfilariae in unstained preparations do not show morphological details sufficiently well to permit identification of species. Stained slides also serve as permanent records of the specimen upon which diagnosis was made.

#### **Giems'a Stained Thick Blood Films:**

##### *Materials and Equipment:*

Blood lancet  
Slide  
Tuberculin syringe with needle  
Applicator stick  
Giemsa's stain (29)  
Buffered water (pH 7.2) (16)

##### *Technique:*

1. To prepare the thick film puncture the finger in the usual manner. As the blood wells up bring a clean slide into contact with the drop of blood. Rotate the slide with a circular motion until an even circle of blood of uniform thickness about the size of a dime has been placed on the slide.
2. Another method for withdrawing blood which will permit taking a large sample is to withdraw 0.1 ml from an arm vein using a tuberculin syringe.

3. To prevent formation of bubbles the last drop is not discharged from the syringe.
4. With a toothpick or applicator stick the blood is then spread in an even film over the greater part of the slide.
5. Thick films should be air dried overnight in a covered container before staining.
6. Lake in tap water for 10 minutes and air dry.
7. Fix in absolute methyl alcohol for 1 minute.
8. Stain in standard Giemsa's stain for 30-45 minutes. (Prepare the stain by mixing 1 ml of stock Giemsa's with 50 ml of buffered water).
9. Differentiate 10 minutes in buffered water.
10. Air dry the slide at room temperature.
11. Preparations are searched under low power and details of organisms found are observed under oil immersion.
12. The body of the microfilariae with the excretory and "G" cells will stain azure. The anal and excretory pores will be reddish pink and the sheath, if present, will be stained light pink. The sheath of *Loa loa* will not stain with Giemsa's.

**Bohmer's Hematoxylin Stain.** The hematoxylin stains are the most efficient for demonstrating the presence or absence of a sheath in microfilariae. Since the presence or absence of a sheath is one of the most diagnostic characteristics in the differentiation of human microfilariae it may often be desirable to utilize Bohmer's hematoxylin stain for this purpose.

##### *Materials and Equipment:*

Bohmer's hematoxylin staining solutions  
A and B (12)  
Distilled water  
Acid Alcohol (add 1 ml of conc. HCl to 100 ml of 70 percent ethyl alcohol)  
(1)

Ammonia water, dilute (1:10,000)  
Alcohol, ethyl, 35, 50, 70, 85, 95 percent  
and absolute  
Xylol  
Balsam, clarite or other suitable mount-  
ing medium  
Slides  
Coverslips

*Technique:*

1. Prepare a thick smear according to method previously described and dry overnight in a covered container.
2. Dehemoglobinize the smear in tap water and air dry.
3. Fix in absolute ethyl alcohol for 1 minute.
4. Cover the smear with stain and heat gently until the smear begins to steam. (To prepare stain add 3 or 4 drops of solution A to 5 ml of solution B just prior to use).
5. Rinse in distilled water.
6. Differentiate in acid alcohol.
7. Rinse in dilute ammonia water.
8. Dehydrate by passing successively through 35, 50, 70, 85, 95 percent and absolute alcohol 5 minutes each.
9. Clear in xylol for 5 minutes and mount in suitable medium.

**Hemalum Stain.** This stain is particularly useful for demonstrating morphological details very clearly. Microfilariae are preserved in their normal attitude.

*Materials and Equipment:*

Hemalum stain (35)  
Normal saline (54)  
Alcohol, ethyl, 15, 35, 70, 85, 95 percent  
and absolute  
Acid alcohol (Add 1 ml conc. HCl to  
100 ml of 70 percent ethyl alcohol)  
(1)  
Xylol  
Distilled water  
Balsam, clarite, or other suitable mount-  
ing medium  
Slides  
Coverslips

*Technique:*

1. Prepare a thick smear according to method previously described and dry overnight in a covered container.
2. Dehemoglobinize dried smears in tap water for 10 minutes.
3. Gradually pass preparations through 15, 35, 50, 70, 85, and 95 percent alcohol, 2-5 minutes each. Transfer to absolute alcohol for 10 minutes for complete fixation.
4. Pass back again through 95, 85, 70, 50, 35, and 15 percent alcohol 2-5 minutes each and then rinse in distilled water.
5. Stain in hemalum stain for a minimum of 5 hours, or overnight.
6. Destain in acid alcohol.
7. Wash slides thoroughly in tap water several times to remove the acid.
8. Pass back up through 15, 35, 50, 70, 85, and 95 percent alcohol, 2-5 minutes each. Dehydrate in absolute alcohol for 10 minutes.
9. Clear in xylol for 10 minutes and mount in clarite, balsam, or other suitable medium.

**Delafield's Hematoxylin Stain:**

*Materials and Equipment:*

Delafield's hematoxylin stain (21)  
Alcohol, ethyl, 95 percent and ether  
(equal parts)  
Acid water (add 0.5 ml conc. HCl to  
100 ml distilled water)  
Balsam, clarite, or other suitable mount-  
ing medium  
Slides  
Coverslips

*Technique:*

1. Make a thick blood film as previously described and dry overnight in a covered container.
2. Lake the smear in tap water and allow to air dry.
3. Fix slides in the alcohol-ether mixture for 10 minutes and again allow to air dry thoroughly.

4. Stain with Delafield's hematoxylin for 10-12 minutes and destain in acid water.
5. Wash in running water until blue color appears in film.
6. Air dry and mount in balsam, clarite, or other suitable mounting medium.

**Methyl Green-Pyronin Stain.** This is a simple technique that gives very good results. The preparation is semi-permanent and lasts at least a year.

*Materials and Equipment:*

Methyl green-Pyronin stain (52)  
 Alcohol, ethyl, 70, 85, 95 percent and absolute  
 Xylol  
 Balsam, clarite or other suitable mounting medium  
 Slides  
 Coverslips

*Technique:*

1. Prepare a thick blood smear as previously described and dry overnight in a covered container.
2. Dehemoglobinize dry smear in tap water for 10 minutes. Air dry.
3. Fix in absolute ethyl alcohol.
4. Transfer smear directly into stain and allow it to remain in the staining solution for 5-15 hours.
5. Pass through 70 percent alcohol 5 seconds, 85 percent alcohol 10 seconds, 95 percent alcohol 15 seconds, absolute alcohol 20 seconds, and a second change of absolute alcohol 1-3 minutes.
6. Clear in xylol for 5 minutes and mount in balsam, clarite, or other suitable medium.
7. Examine the preparation under low power and observe morphological details under oil immersion. The ordinary cells of the nuclear column stain green, the excretory and anal pores and special cells stain red, while cuticle and sheath stain gray.

## Concentration of Microfilariae in Blood

In cases where small numbers of worms are present, the centrifugation of citrated or heparanized blood, or the centrifugation of blood in which the erythrocytes have been laked out is a most efficient technique for recovery of microfilariae. In suspected cases of filarial infection at least one of the techniques here described should be performed if more direct methods of examination prove negative.

**Examination of Citrated Blood:**

*Materials and Equipment:*

Sodium citrate, 2 percent solution in physiological saline  
 Centrifuge tubes  
 Syringe, 10 ml, with needle  
 Capillary pipette  
 Slides  
 Coverslips

*Technique:*

1. Draw 5 ml of blood by venipuncture and add to 1 ml of sodium citrate solution in centrifuge tube and mix well.
2. Centrifuge at 1000 rpm for 10 minutes. Three layers will form consisting of a large well packed red cell layer in the bottom of the tube, a thin creamy leucocyte layer above the red cells, and an uppermost straw colored layer of supernatant fluid at the top of the tube.
3. With a capillary pipette carefully draw off the middle leukocyte layer and place it on one end of a slide and coverslip. With the same pipette reach to the very bottom of the tube and draw a small amount of the red blood cell sediment. Place on the opposite end of the same slide and coverslip.
4. Examine both preparations under low power, thoroughly searching the entire area of the coverslips for actively moving microfilariae. Confirm findings under high dry.

### **Examination of Heparinized Blood:**

#### *Materials and Equipment:*

Heparin, 4 percent solution in physiological saline  
Centrifuge tube  
Syringe, 10 ml, with needle  
Capillary pipette  
Slides  
Coverslips

#### *Technique:*

1. Prepare centrifuge tubes in advance by placing 0.25 ml of the heparin solution in each tube. Evaporate to dryness in a 37° C. incubator. (Each tube will contain 10 mg of heparin.)
2. Obtain 5 ml of blood by venipuncture, add it to the tube and mix well.
3. Proceed with step 2 of technique described above for citrated blood.

### **Examination of Lake Blood**

Hemolyzing blood offers the very distinct advantage of removal of a large portion of the sediment, thus increasing the chances of finding microfilariae when they are present in small numbers. Various reagents including dilute formalin, acetic acid, and saponin can be used to hemolyze the red cells. The disadvantage of laking blood with dilute formalin or acetic acid is that the organisms are killed and their presence is not revealed by their characteristic motion. This disadvantage is overcome when saponin is used to lake the cells since with this reagent the microfilariae remain in an active motile condition.

#### **Knott's Technique:**

#### *Materials and Equipment:*

Syringe, 2 ml, with needle  
Centrifuge tube  
Formalin, 2 percent solution, or acetic acid, 2 percent solution  
Capillary pipette  
Alcohol, 95 percent, and ether mixture (equal parts)  
Buffered water (pH 7.2) (16)  
Giemsa's stain (29)

Slides

Coverslips

#### *Technique:*

1. Draw 1 ml of blood by venipuncture and mix it with 10 ml of a 2 percent solution of formalin or acetic acid.
2. Centrifuge for 5 minutes at 1,500 rpm.
3. Pour off the supernate without disturbing the sediment. With a capillary pipette transfer a portion of the sediment to a clean slide.
4. Apply a coverslip and examine the whole preparation under low power. Confirm findings under a high dry. If objects known or suspected to be microfilariae are found proceed with step 5.
5. Smear the remaining sediment over a slide. Air dry and fix in the alcohol-ether mixture for 10 minutes. Remove the smear and air dry.
6. Stain with Giemsa's diluted 1-50 with buffered water for 30-50 minutes followed by destaining with buffered water for 10 minutes.
7. Air dry the smear and examine under low power, confirming findings under high dry.

### **Examination of Saponin Hemolyzed Preparations:**

#### *Materials and Equipment:*

Syringe, 2 ml with needle  
Saponin, 0.2 percent solution  
Centrifuge tube  
Capillary pipette  
Slides  
Coverslips

#### *Technique:*

1. Draw 1 ml of blood by venipuncture and add it to a centrifuge tube containing 10 ml of the saponin solution.
2. Mix thoroughly and centrifuge at 1,500 rpm for 10 minutes.

3. Pour off the supernatant fluid without disturbing the sediment and with a capillary pipette transfer the sediment to a clean slide.
4. Apply a coverslip and examine for active microfilariae under the low power of the microscope, confirming findings under high dry.

## SHIPMENT OF BLOOD SPECIMENS FOR MICROFILARIAL DIAGNOSIS

Smaller Army laboratories are often not equipped to perform laboratory procedures for the detection of microfilarial infection in blood. In such situations it will be necessary to forward specimens to reference laboratories for examination. Three methods of preparation of specimens for shipment are as follows:

1. Thick blood films may be prepared and forwarded for staining and examination.
2. Blood may be drawn in 5 ml quanti-

ties and treated with anticoagulant. One ml of sodium citrate, 2 percent solution, in physiological saline, or 10 mg of heparin dried in the bottom of a test tube can be used for this purpose. Microfilariae will remain alive for several days when blood is treated in this manner.

3. Blood may be preserved by addition of 10 ml of 2 percent formalin to 1 ml of blood.

## LABORATORY DIAGNOSIS OF ONCHOCERCIASIS

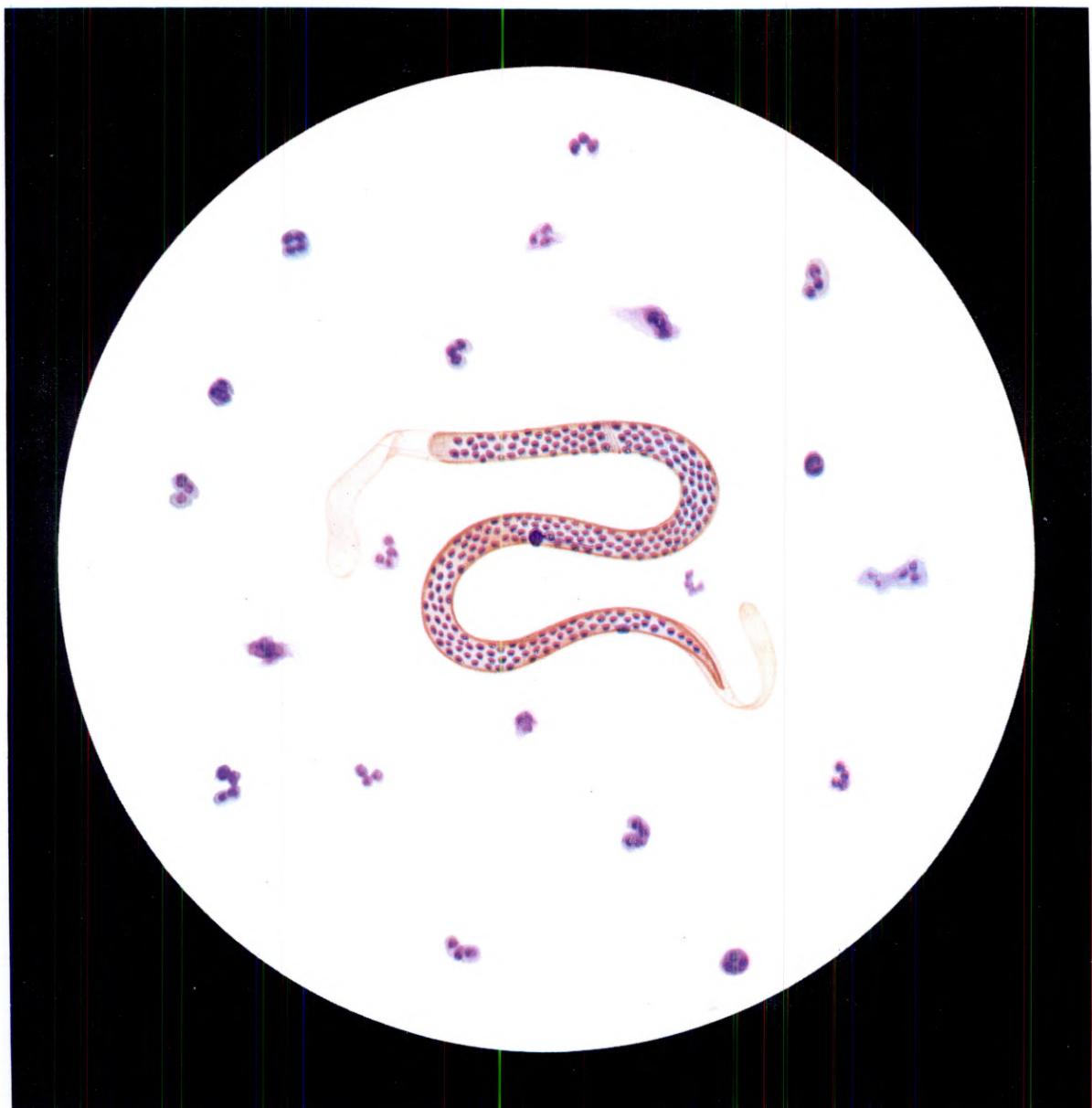
Onchocerciasis may be diagnosed either by demonstration of the microfilariae in fluid aspirated from a nodule or by their recovery from small sections of skin excised with a sharp instrument at or near the site of the nodule. The excision should be so superficial that no blood is drawn. Excised tissue is mounted in warm normal saline and cover-slipped. The preparation should be allowed to stand for a few minutes and then observed under high dry magnification. If living microfilariae are present they will soon be observed

to migrate out of the tissue section into the surrounding fluid. Since some nodules may contain only adult worms or dead larvae, failure to find the microfilariae does not indicate absence of infection. If ocular lesions are present, the embryos may be demonstrable in tissue removed from the bulbar conjunctiva. Occasionally microfilariae may be found in the skin when there are no palpable nodules, and rarely the microfilariae can be recovered in peripheral blood. The latter cases usually show a rather marked eosinophilia.

**Table 1. A Comparative Table of the Medically Important Filarial Worms**

Diagnostic Characteristics	<i>W. bancrofti</i>	<i>W. malayi</i>	<i>O. volvulus</i>	<i>A. perstans</i>	<i>M. ozzardi</i>	<i>Loa loa</i>
Geographical distribution.	Roughly the entire region of the world lying between the Tropic of Capricorn and the Tropic of Cancer.	Malaya, the East Indies, the Celebes, India, Indo-China, Ceylon and South China.	Circumscribed areas of Southern Mexico and Guatemala.	South America as far south as Argentina; extensively in regions of Africa.	Central and South America and the West Indies.	West and Central Africa, particularly the Congo River and its tributaries.
Sheath	Sheath present-----	Sheath present-----	No sheath-----	No sheath-----	No sheath-----	Sheath present.
Cephalic space	As long as broad-----	Twice as long as broad.	-----	-----	-----	As long as broad.
Nuclear column in tail.	Nuclei not extending to tip of tail.	Nuclear column terminating in two distinct nuclei. Tail somewhat swollen.	Tail sharply pointed, and gracefully curved, nuclei not reaching tip of tail.	Tail blunt.	Nuclei extending to tip of tail.	Nuclei extending to tip of tail.
Location within host.	Peripheral blood, hydrocoele fluid.	Peripheral blood-----	Subcutaneous nodules.	Blood, principally in large arteries.	Peripheral blood-----	Peripheral blood.
Periodicity	Usually nocturnal-----	Nocturnal but less absolutely so than <i>W. bancrofti</i> .	Non-periodic-----	Non-periodic but microfilariae more numerous nocturnally.	Non-periodic-----	Diurnal.
Intermediate arthropod host.	Mosquitoes (several general).	Mosquitoes (several general).	Black flies ( <i>Simulium</i> sp.).	Midges ( <i>Culicoides</i> sp.).	Midges ( <i>Culicoides</i> sp.).	Tabanid flies ( <i>Chrysops</i> sp.).
Host Relationships	Distinguishing Morphology					





*Wuchereria bancrofti* in lactated blood.



# PART THREE

## HISTOLOGIC AND SERODIAGNOSTIC EXAMINATIONS

### Chapter 7

#### DIAGNOSIS OF PARASITIC INFECTIONS BY TISSUE EXAMINATION

Properly chosen tissue specimens are often the key to solving a diagnostic enigma. Not only can they afford the information necessary to pin down the true nature of a parasitic infection but they can conclusively separate other diseases that may be confused with parasitic diseases. There are some types of parasitism (e.g., sparganosis, echinococcosis) in which diagnosis can only be established by tissue examination. There has been a hesitancy in many situations to resort to biopsy. Undiagnosed patients with enlarged superficial lymph nodes which contain diagnostic

lesions are permitted to go for months without biopsy, and yet removal of a superficial lymph node is no more formidable a procedure than incision of an abscess. Skin biopsy, bone marrow aspiration and biopsy of accessible mucous membranes are also safe procedures in the hands of trained physicians. Needle biopsy of spleen, liver, lung and other organs is not without hazard but is safe enough in experienced hands that there should be no hesitation when such a procedure is necessary to establish the diagnosis.

#### PROTOZOAN INFECTIONS

##### **Protozoal Dysentery**

Where stool examinations prove disappointing in suspected cases of amoebic dysentery, proctoscopic biopsy may be revealing. The discrete, deep ulcers of amoebic colitis can be identified by direct observation through the proctoscope or sigmoidoscope. Small pieces of tissue are removed from the base or edge of ulcers by means of special biopsy forceps. The tissue is divided into two portions. One portion is fixed in 10 percent formalin and processed for H and E stained tissue sections. The pathologist will recognize the typical flask-shaped ulcers and the trophozoites of *E. histolytica*. The other portion of tissue is macerated in a few drops of warm saline (37° C.) with sharp scissors. Part of this material can be observed by direct saline or

vital stained preparation for typical trophozoites. The procedure to be followed is described in chapter 1. Rarely *Balantidium coli* ulcers are encountered in the large intestine of humans. They are studied the same as for *E. histolytica* except that they cannot be cultured. Needle biopsies of amoebic liver and lung abscesses are treated the same way as intestinal ulcers. The trophozoites are most abundant in the granulation tissue in the shaggy portion of the wall of the abscess. *E. histolytica* occurs in the appendix in about 50 percent of cases. Cysts of *E. histolytica* do not occur in the tissue lesions.

##### **Malaria, Trypanosomiasis and Leishmaniasis**

For the present, at least, biopsy is of limited value in the diagnosis of malaria. Needle

biopsy of enlarged liver and spleen may demonstrate typical malaria pigment. Blood smears are generally superior to tissue biopsy in demonstration of parasites.

Lymph node biopsy may be of definite value in African trypanosomiasis, especially early in the disease when lymph nodes are enlarged. The trypanosomes can be demonstrated by culture, animal inoculation, H and E or Giemsa's stained tissue sections, and stained or unstained smears of material obtained by macerating a portion of a lymph node.

In South American trypanosomiasis recovery of parasites from the peripheral blood may be very difficult. The organisms localize in the heart and brain. Enlarged lymph nodes and "chagomas" (nodular formations at the site of the bite of the arthropod) may harbor organisms. These materials are examined for

the presence of organisms by maceration and preparation of Giemsa's stained smears and inoculation into mice or guinea pigs.

In *Leishmania donovani* infections the organisms are rarely found in the cells of the circulating blood. However, tissue biopsy is an especially gratifying diagnostic procedure. The organisms are usually easily demonstrated in Giemsa's or Wright's stained smears of bone marrow. Spleen, liver and lymph node tissue usually contain the organisms. The organisms are revealed in these tissues by stained smears or tissue sections. Material from any of the above sources can be used for culture or hamster inoculation. In cutaneous and mucocutaneous leishmaniasis the intracellular parasites can be demonstrated in stained smears and sections of tissue taken from the skin and mucous membrane lesions.

## NEMATODES

### Intestinal Roundworms

*Trichuris trichiura* and *Enterobius vermicularis* adults may be found in the appendix. The latter is a fairly common finding in appendices removed for appendicitis or incidentally in the course of other abdominal operations. *Ascaris lumbricoides* may also be found in the appendix. A lesion in the wall of the appendix called "eosinophilic granuloma", the most conspicuous feature of which is large numbers of eosinophilic leukocytes, may be caused by the ova or adults of any of the intestinal worms, especially the three listed above.

Hookworm adults in the intestine are not ordinarily accessible for biopsy. However, if adults are present there will be ova in the stools and biopsy would be of no special value. Creeping eruption is caused by non-human species of hookworm. The rhabditiform larvae can be identified in H and E stained sections of tissue taken from the advancing end of the creeping skin eruption.

### Tissue Roundworms

*Trichinella spiralis* larvae are most readily demonstrated in skeletal muscle tissue during

the third or fourth week after the onset of symptoms. Best results are obtained with tissue taken from the deltoid, biceps or gastrocnemius muscles. The muscle tissue can be handled as follows: (1) Macerate a portion of the muscle tissue and digest in artificial gastric juice for several hours at 37° C. Place a few drops of the sediment on a slide, cover-slip and examine for larvae. (2) Cut a portion of the muscle into thin slices approximately 1 mm in thickness. Compress tightly between two glass slides using rubber bands to maintain tight compression. Examine under the microscope with proper lighting for encysted larvae. (3) Prepare H and E stained tissue sections. Examine for encysted larvae.

Since 1947 a parasitic syndrome has been recognized and designated "visceral larva migrans" because of certain similarities to larvae migrations of the skin called cutaneous larva migrans. This syndrome characteristically has its onset in infants and young children under the age of three and is characterized by leukocytosis (usually over 20,000/cu mm), eosinophilia (over 20 percent), enlarged liver, hyperglobulinemia and benign course. The evidence to date indicates that the syndrome is due in most if not all cases to invasion of

the liver by nematode larvae. In the reported cases in which the etiologic agent has been positively or tentatively identified, either *Ascaris lumbricoides* or *Toxocara canis* has been implicated. Repeated stool examinations are frequently negative for ova. Liver biopsy shows scattered grayish-white granulomas up to several mm in diameter. Microscopically the nematode larvae can be identified in the midst of the granulomatous foci. Specific identification may require careful examination of the larvae in step serial sections, taking note of length, diameter, and details of morphology. As more cases are reported and additional information is accumulated concerning the morphological characteristics of nematode larvae in tissue sections, identification should become simplified. Needle biopsy may be tried; if this fails it may be necessary to resort to laparotomy and removal of grossly visible granulomas under direct vision.

For further details concerning the clinical and pathological features of visceral larva migrans the reader is referred to the excellent case report and review by Milburn and Ernst, Pediatrics 11:358, Apr. 1953.

### Filariasis

As a rule careful and persistent examination of the peripheral blood for microfilariae is the most rewarding procedure in the diagnosis of the more common filarial infections. Indeed, microfilariae are commonly demonstrable in the blood long before there are any clinical manifestations or localizing signs. However, it may be necessary in certain cases to resort to tissue biopsy to demonstrate the etiologic agent. Although microfilariae are ordinarily demonstrable in the blood before there is evidence of localizing signs, the reverse situation may occur; namely, lymph node enlargement as early as three months after infection before the worms have had time to reach sexual maturity and, therefore, before they are capable of producing microfilariae. In these cases lymph node biopsy already shows the characteristic lesions and developing (though non-gravid) female filariae and developing male filariae.

The adult worms of *Wuchereria bancrofti* tend to localize in the lymph nodes of the groin and in the epididymis. Sections prepared from excised lymph nodes and biopsies of involved epididymis show the males and gravid females in the lymphatic vessels with accompanying reactive phenomena. In *W. malayi* infection the region of the breast may be involved in the female in addition to the sites noted above. The elephantiasis that occurs as a late complication in the lower extremities, scrotum, vulva and breast displays rather characteristic microscopic features consequent to prolonged lymph stasis but demonstration of the adult etiologic agents is not to be expected. *Acanthocheilonema perstans*, an uncommon filaria of man localizes in the mesentery, pleura, pericardium and liver. Nodular lesions should be selected for section and microscopic examination. *Mansonella ozzardi*, another uncommon filaria of man, also localizes in the mesentery and peritoneum. There are no clinical manifestations and little tissue reaction is provoked. Both of the latter species produce microfilariae in the peripheral blood without periodicity.

*Loa loa* characteristically produces two types of lesions which are easily accessible for biopsy and in which the adults can frequently be recovered. One is the so-called "Calabar" swelling, a transient subcutaneous nodule produced by the migrating adults, any particular nodule lasting only 2 or 3 days. The other is localization beneath the conjunctiva. The worm is not always found in the subcutaneous swellings since it may have already departed to continue its migration before the biopsy is taken. The adults are 3-7 cm in length by approximately 0.5 mm in diameter and they can easily be visualized and removed for identification when they localize beneath the conjunctiva.

Both microfilariae and adults can be demonstrated in skin biopsies of *Onchocerca volvulus* infections. Microfilariae may be found in large numbers in the skin, sometimes even in biopsies taken from areas which appear normal to the naked eye. It is preferable, however, to select biopsies from obviously inflamed and lichenified areas to demonstrate the micro-

filariae. The latter are most concentrated in the superficial dermis and the dermal papillae. The microfilariae are photophilic and are prone to migrate to the eye, particularly when there are nodules in the scalp. They invade the cornea first, and later the interior of the eye. Blindness may result. The adults are to be found in subcutaneous nodules which measure up to several cm in diameter. These nodules are well circumscribed but usually not truly encapsulated. The center may be soft, cheesy, yellow or honeycombed. Often several male and female adult worms are found inextricably bound into a mass occupying the center of the nodule. The surrounding connective tissue shows inflammatory and reactive changes and there are frequently large numbers of microfilariae in the vicinity. The nodules are likely to occur on the trunk in African cases and the scalp in Central American cases. The nodules resemble the well known sebaceous cyst.

### **Dracunculiasis**

*Dracunculus medinensis* is not a true filarial worm and the extruded larvae do not inhabit the human host. Characteristically the adult female produces a blister in the skin of the lower extremity and then a tortuous tract forms in the adjacent skin outlining the worm. The larvae can be demonstrated by placing the blister in contact with water, permitting the blister to rupture, and collecting the larvae. The live worm can be dissected out of the tract prior to rupture of the blister. Death of the female is followed by moderate local inflammatory and foreign body reaction followed by fibrosis and calcification. In the later stages it would probably not be possible to dissect out the worm intact; block dissection of the involved tissue would be necessary. The vesicles and worm tracts are most common on the soles of the feet and ankles, but may occur on the leg, thigh, knee, upper extremity, scrotum or trunk.

## **TREMATODES**

### **Paragonimiasis**

Biopsy procedures are more important in the diagnosis of *Paragonimiasis* and *Schistosomiasis* than in *Clonorchis*, *Fasciolopsis*, or *Fasciola* sp. infections. In *Paragonimus westermani* infection the parasites may encyst in the intestinal wall, peritoneum, diaphragm or pleura failing to complete their migration to the lung. In these cases the ova do not have access to the respiratory tract and diagnosis can only be established by biopsy. Occasionally fistulous tracts are established between abdominal cysts and the intestines or skin. In these cases it may be possible to recover ova in the stool or skin discharge respectively. In the absence of communication with a surface, diagnosis depends on removal of one of the bluish cysts from the intestine, peritoneum, diaphragm or pleura and identification of the adults and ova by teased preparations and microscopic sections.

Usually in pulmonary paragonimiasis the ova are present in the sputum. If the disease is suspected and ova are not demonstrable in

the sputum, needle aspiration of one of the subpleural cysts may reveal the typical ova. Lymph node, skin, or brain involvement may occur as the result of ectopic migration in paragonimiasis. Cysts or abscesses formed in these sites are examined for ova and adults by smears, teased preparations and microscopic sections.

### **Schistosomiasis**

In schistosomiasis infections biopsy procedures are very helpful when ova are not found in the stool or urine. The purpose of biopsy is to demonstrate the characteristic ova; the adults will usually not be obtained. When biopsy is rewarded by the finding of adults, the most reliable criterion for the identification (exclusive of the characteristics of the ova) is the appearance of the integument on cross section. *S. mansoni* shows coarse tubercles; *S. hematobium*, fine tubercles; and *S. japonicum*, a smooth non-tuberculate integument. Biopsies intended to recover the diagnostic ova should be selected from granular,

eroded, or polypoid areas in the mucosa of the rectum (via proctoscopy) and bladder (via cystoscopy). The tissue obtained may be divided, one part being processed for tissue sections and the other part cut into fine pieces with scissors, macerated in saline, and un-

stained slides prepared for identification of the characteristic ova of one of the three species. Needle biopsy of the liver may reveal pseudotubercles containing ova in the portal areas. Schistosome ova are also occasionally deposited in the lung and brain.

## CESTODES

Biopsy methods are the definitive procedures for the diagnosis of the larval stages of certain tapeworm infections of man; namely, sparganosis, cysticercosis and echinococcosis.

### Sparganosis

Sparganosis of man refers to infection with the plerocercoid larval stage of *Diphyllobothrium latum*, *D. mansoni* (adults in dogs and cats) and "Sparganum proliferum," a form in which the adult stage has not yet been identified. The sparganum migrates and finally localizes in skeletal muscle, peritoneum or orbit. The localized larvae frequently incite the formation of purulent abscesses. Infection with *S. proliferum* is more serious because the larvae form branches which detach, migrate to new sites, enlarge, branch, and so on until thousands of larvae are present in various tissues of the body. Diagnosis of sparganosis depends on removal of nodule or abscess from the skin, muscle or other accessible site, dissection of the tissue, and removal of the sparganum larva. The larva (which is 1, 2 or more cm in length by 2 or more mm in diameter) should be removed intact if possible, then fixed, flattened and stained by one of the procedures described for cestodes. The specimen can then be identified by comparing its morphological characteristics with the descriptions in standard references on parasite morphology.

### Cysticercosis

Man occasionally serves as an accidental intermediate host for "Cysticercus cellulosae," the larval form of *Taenia solium*. The cysticercus may localize in any tissue in the body, particularly voluntary muscles, subcutaneous

tissues, brain (especially meninges and ventricles), and orbit. The fully developed cysticercus or "bladder worm" is about 1 cm in diameter. The live bladder worm lies relatively free in the tissue with little reaction. After death of the parasite there is an inflammatory reaction around the cysticercus followed by fibrosis and calcification. The living bladder worm obtained from a muscle or subcutaneous nodule or other site of localization consists of a thin walled cyst approximately 1 cm in diameter with a smooth inner surface, filled with clear fluid, and containing a grossly visible invaginated scolex. H and E sections show characteristic features of bladder wall and scolex. Dead worms are not as typical but the gross and microscopic features are usually sufficiently characteristic to be diagnostic.

### Echinococcosis

Hydatid cyst represents the larval form of *Echinococcus granulosus*. Approximately 70 percent of hydatid cysts occur in the liver, about 15 percent in the lungs and the remainder in bone, brain and other organs. The cysts grow very slowly and by the time they are manifested the infection has probably been present for several years. Several morphologic varieties of hydatid cysts occur; namely, simple sterile unilocular cysts; fertile unilocular cysts with endogenous (internal) and/or exogenous (external, in surrounding tissues) daughter cysts; multilocular, nonencapsulated sterile alveolar cysts which have a honeycomb appearance; and hydatid cysts in bones, which usually occur at the epiphyseal end and are not spherical but infiltrate between the bone trabeculae in the confines of natural barriers

to growth. Great care should be taken to remove cysts in toto wherever possible, for rupture of a cyst will result in spillage of cyst contents into surrounding tissue thus setting up multiple new centers of cyst formation. For this reason needle biopsy or removal

of small pieces of cyst for diagnosis is generally contraindicated. The typical laminated membrane, scolices and hooklets of echinococcus cysts are easily identified in H and E stained tissue sections and smears or wet mounts of cyst fluid.

## Chapter 8

# DIAGNOSIS OF PARASITIC INFECTIONS BY SEROLOGICAL METHODS

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Immunologic methods for parasitic infections include intradermal, precipitin, and complement-fixation tests. Most of the antigens are not commercially available and must be prepared in the laboratory. Many of the antigens to be described are only group specific and the results of tests in which these antigens are utilized require careful interpretation. Fortunately, it is usually not necessary to resort to immunologic procedures because the causative parasites can ordinarily be demonstrated

by careful examination of readily available specimens. However, in some types of parasitism (i.e., cysticercosis, abdominal paragonimiasis, abdominal schistosomiasis, certain filarial infections, and trichinosis) serologic tests may be very helpful.

The more useful immunologic procedures are described in detail in the section which follows. A summary of serologic tests for parasitic diseases is given in table 1.

## AMOEBIASIS

### Complement-Fixation Test

(Bozicevich, Hayem, and Walston)

#### REAGENTS:

1. Two percent sheep cell suspension.
  - a. Filter the required quantity of sheep's blood through gauze into a 50 ml centrifuge tube. Add 2-3 volumes of saline solution. Centrifuge at a speed of 1,800 rpm in a No. 1 International centrifuge (1,530 rpm in a No. 2 centrifuge) for 5 minutes.
  - b. Remove the supernatant fluid and thin upper layer of white cells by suction or a capillary pipette.
  - c. Resuspend the corpuscles in 3 or 4 volumes of saline by gentle shaking and again centrifuge. Repeat this procedure three times. The last time, rather than resuspending the corpuscles, they should be washed into a graduated centrifuge tube. If the supernate is not colorless after three washings, the corpuscles are too fragile and should not be used.

- d. Centrifuge the graduated centrifuge tube containing sheep corpuscles at 2,000 rpm in a No. 1 International (1,700 rpm in a No. 2) for 10 minutes to pack the corpuscles. This speed and time interval should be kept constant from day to day in order to obtain comparable 2 percent suspensions. To prepare the 2 percent suspension, add 49 volumes of chilled saline per volume of *packed* corpuscles. *Do not* prepare the 2 percent suspension by withdrawing and measuring sheep corpuscles with a pipette. Store the 2 percent suspension in the refrigerator at 6°-8° C. when not in actual use. Always mix the suspension of corpuscles well before using to insure uniformity per unit volume. A fresh sheep cell suspension should be prepared daily.
2. Hemoglobin standard
  - a. Pipette 10 ml of 2 percent cell suspension into a graduated centrifuge tube. Centrifuge at 2,000 rpm for 10 minutes.

- b. Withdraw as much of supernate as possible with a capillary pipette without disturbing the cells.
- c. Add distilled water up to the 9.5 ml mark.
- d. Lake the cells by shaking.
- e. Add 0.5 ml of a 17 percent saline solution to restore isotonicity. This is the hemoglobin standard.

**Color Standards**

Percent of Hemolysis	0	10	20	30	40	50	60	70	80	90	100
Saline (0.85 per cent).....	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Hemoglobin Standard.....	0.00	0.02	0.04	0.06	0.08	0.10	0.12	0.14	0.16	0.18	0.20
2 percent red cells.....	0.20	0.18	0.16	0.14	0.12	0.10	0.08	0.06	0.04	0.02	0.00

**3. Titration of hemolysin**

- a. Make up hemolysin dilutions of 1:2000 to 1:7000. Dilutions are prepared from a stock solution of 1:100 hemolysin as follows:

Phenol (5 percent in saline). 4 ml  
 Saline solution..... 94 ml  
 Glycerinized hemolysin (50 percent)..... 2 ml  
 Phenol and saline are mixed before addition of glycerinized hemolysin to eliminate the possibility of precipitation of hemolysin by 5 percent phenol.  
 1.0 ml hemolysin (1:100) + 19.0 ml saline=1:2000  
 6.0 ml hemolysin \*(1:1000) + 9.0 ml saline=1:2500  
 5.0 ml hemolysin (1:1000) + 10.0 ml saline=1:3000  
 3.0 ml hemolysin (1:1000) + 7.5 ml saline=1:3500  
 3.0 ml hemolysin (1:1000) + 9.0 ml saline=1:4000  
 6.0 ml hemolysin (1:2000) + 7.5 ml saline=1:4500  
 5.0 ml hemolysin (1:2500) + 5.0 ml saline=1:5000  
 2.0 ml hemolysin (1:1000) + 9.0 ml saline=1:5500

\*Prepare 1:1000 hemolysin by mixing 2.5 ml of 1:100 hemolysin with 22.5 ml saline.

5.0 ml hemolysin (1:3000) + 5.0 ml saline=1:6000  
 2.0 ml hemolysin (1:1000) + 11.0 ml saline=1:6500  
 3.0 ml hemolysin (1:2000) + 7.5 ml saline=1:7000

- b. Ten ml of each dilution of hemolysin is mixed with equal volume of 2 percent cell suspension. This is allowed to stand for at least 15 minutes before using. The mixture of hemolysin and cells represents the "sensitized cells" for each hemolysin dilution.
- c. Prepare a 1:100 dilution of complement.
- d. Arrange the tubes for the hemolysin titration in 11 rows of 10 each. Add the following amounts of saline and 1:100 complement to the respective tubes of each row.

Tube Numbers										
1	2	3	4	5	6	7	8	9	10	
Saline.....	0.55	0.50	0.45	0.40	0.35	0.30	0.25	0.20	0.15	0.10
Complement....	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45	0.50

Each tube now contains 0.6 ml. Next add 0.4 ml of "sensitized cells" to all tubes. In the first row 0.4 ml of "sensitized cells" made from 1:2000 hemolysin dilution is added to each tube. In the next row 0.4 ml of "sensitized cells" made from 1:2500 hemolysin dilution is added to each tube. This is continued, using the next consecutive dilution for each successive row.

- e. Place the racks in a 37° C. water bath for 30 minutes, remove and centrifuge the tubes at 2000 rpm for 10 minutes (centrifuge the 10 color standard tubes at the same time). Compare each tube with the color standard tubes and record the results in percent hemolysis. The following table is an example:

Hemolysin Dilutions	Tube Numbers									
	1	2	3	4	5	6	7	8	9	10
1:7000.....	0	0	0	0	5	10	20	25	35	45
1:6500.....	0	0	0	8	20	30	40	55	65	68
1:6000.....	0	0	3	10	25	35	50	60	65	70
1:5500.....	0	0	5	15	35	45	55	70	80	85
1:5000.....	0	0	8	30	50	60	80	85	90	95
1:4500.....	0	0	20	40	60	70	85	88	95	98
1:4000.....	0	0	25	50	75	80	85	90	95	100
1:3500.....	0	10	30	60	80	85	95	100	100	100
1:3000.....	0	30	55	80	90	95	98	100	100	100
1:2500.....	0	35	60	85	93	98	100	100	100	100
1:2000.....	2	37	65	90	95	100	100	100	100	100

The optimal dilution of hemolysin is defined as that dilution beyond which further increase in the concentration of hemolysin does not appreciably change the quantity of complement required for 50 percent hemolysis. In the example given, then, the optimal dilution of hemolysin would be 1:3000. This dilution is used henceforth for the daily complement titration and for the preparation of sensitized cells for the test proper.

#### 4. Titration of complement

- On the day that the hemolysin is titrated, the hemolysin titration also serves as the complement titration, and in this instance a separate complement titration is not necessary.
- It is mandatory that the complement titration be done daily, using the optimal dilution of hemolysin as originally determined. Prepare a 1:100 dilution of complement. Titrate according to the following table:

	Tube Numbers										
	1	2	3	4	5	6	7	8	9	10	11
Saline.....	0.55	0.50	0.45	0.40	0.35	0.30	0.25	0.20	0.15	0.10	
Complement (1:100)....	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45	0.50	
Sensitized cells.....	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	

Incubate in a 37° C. water bath for 30 minutes, remove, centrifuge the tubes at 2,000 rpm for 10 minutes and compare with the color standards as before. (The color stand-

ard must be made fresh daily.) The tube which matches exactly the 50 percent color standard is the exact unit of complement. If the 50 percent end point falls between two tubes, it is necessary to interpolate the exact unit of complement. Since 4 exact units are required for each 0.2 ml to be used in the test, use the following formula: (In this example, assume that the 50 percent end point was tube 3, containing 0.15 ml of diluted complement.)

$$4 \times 0.15 = 0.60 \text{ ml of 1:100 complement}$$

↑  
amt. of diluted complement causing 50 percent end point

$$\text{Therefore } \frac{0.60}{100} = \frac{0.20}{x}$$

$$x = 33.3$$

Therefore 0.2 ml of 1:33.3 dilution of complement will be required for the test. (One ml complement plus 32.3 ml of saline.)

#### 5. Antigen

The only reliable antigen generally available is the *Endamoeba histolytica* antigen prepared by Hynson, Wescott, and Dunning, Inc., Baltimore, Md. The antigen is supplied in 5 ml ampules, packaged in boxes of five.

#### PROCEDURE FOR TEST:

- Inactivate serum at 56° C. for 30 minutes just prior to use.
- Set up the test as follows:

	Tube Numbers										
	1	2	3	4	5	6	7	8	9	10	11
Saline.....	0.30	0.25	0.20	0.30	0.25	0.20	0.30	0.25	0.20	0.30	
Serum 1:5.....	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	
Serum 1:10.....	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	
Complement (4 units/0.2 ml)....	0.20	0.20	0.10	0.15	0.20	0.10	0.15	0.20	0.10	0.15	0.20
Antigen.....	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	

- Place tubes in refrigerator overnight (15-18 hours) at 5°-8° C.
- The next day add 0.4 ml of sheep cells which have been sensitized 15 minutes previously.

- Incubate in 37° C. water bath for 30 minutes.
- Remove and read.

#### INTERPRETATION:

- This test takes into consideration the amount of binding of complement by the serum dilutions and the antigen. From the table for the test procedure above, one will note that tubes 3, 4, and 5 contain, respectively, 2, 3, and 4 fifty-percent-units of complement to determine the binding power of the 1:5 serum dilution. Tubes 6, 7, and 8 are devised in an identical manner to determine the binding power of the 1:10 serum dilution. Tubes 9, 10, and 11 determine the binding power of the antigen. It is necessary to determine binding powers for every serum used in the test, but only one set of binding powers need be conducted on the antigen for any particular day.
- Remove the tubes containing 30, 50, 70, and 100 percent *cells* (not percent hemolysis) of the color standard and resuspend the cells. Any tube in the test which matches the tube containing 30 percent or more is positive. Any tube showing less than 30 percent cells is negative.
- Examples:
  - Negative test

Tube Numbers											
1	2	3	4	5	6	7	8	9	10	11	
1:5 serum..	++++	++++	+++	-							
1:10 serum..	+				++++	+	-				
Antigen...					-	-	-				

In this example tubes 3, 4, and 5 show that the serum itself binds 3 units of complement. Yet with the

usual methods, tube 5 would be the control tube and the test would be regarded as satisfactory. The 1:10 dilution of serum also has some binding power as shown in tube 7. This serum is regarded as negative.

#### b. Poor antigen

Tube Numbers											
1	2	3	4	5	6	7	8	9	10	11	
1:5 serum..	++++				-	-	-				
1:10 serum....		++++				-	-	-			
Antigen...						++++	++++	++	-		

Tubes 9 and 10 disclose that the antigen binds at least 3 units by itself. Tube 11, the usual control in other tests, is negative and would be regarded as a satisfactory antigen control. Result: test unsatisfactory. Must be repeated with new antigen.

#### c. Positive test

Tube Numbers											
1	2	3	4	5	6	7	8	9	10	11	
1:5 serum..	++++				++++	++	±				
1:10 serum....		++++				-	+++	±	-		
Antigen...							-	-	-		

This test is considered positive because there is a good fixation in the 1:10 dilution, and in this dilution the anticomplementary effect of the serum is practically removed (tube 7). The 1:5 dilution is unsatisfactory because of the strong anticomplementary effect.

- For further clinical interpretation, see table 1, Immunologic Tests for Parasitic Diseases.

## VISCERAL LEISHMANIASIS

### Aldehyde Test (Napier)

#### PROCEDURE:

- To a test tube containing 1 ml of patient's serum, add 1 drop of 40 percent formaldehyde. Shake well.

- Allow to stand at room temperature.
- If the serum becomes opaque and gels within 3-30 minutes, the test is positive. If the test is negative, no reaction should occur in 24 hours. The

appearance of opaque serum which does not gel is a doubtful reaction. This may occur in early infections.

#### INTERPRETATION:

1. This is a nonspecific test for increased serum globulin. It is positive in 82 percent of cases of Kala-azar.
2. False positive tests occur in any condition in which serum globulin is elevated (e.g., schistosomiasis).
3. Doubtful reactions require confirmation by other diagnostic procedures.

### Antimony Test (Chopra)

#### PROCEDURE:

1. Place 0.2 ml of undiluted serum in one tube and 0.2 ml of 1:10 dilution of serum in a second tube.

2. Carefully overlay the contents of each tube with a 4 percent solution of pentavalent antimony (ureastibamine).
3. The appearance of a thick flocculent disc at the interphase is a positive test.

#### INTERPRETATION:

1. At the height of the infection, the test shows a positive reaction almost immediately. In early infections, the reaction may only be positive in the undiluted serum and/or the reaction may be delayed for a few minutes to an hour.
2. After effective treatment, the test becomes negative.
3. The test is positive in 88 percent of cases of visceral leishmaniasis.

## TRICHINOSIS

### Intradermal Test

#### PREPARATION OF ANTIGEN:

1. To 80 gm of muscle tissue (cut into small pieces) from freshly sacrificed infected rats, add 1,500 ml of a 0.6 percent pepsin-0.3 percent HCl solution. Digest at 37° C. for 12 hours. Agitate periodically.
2. Filter through 6 layers of cheesecloth.
3. Dilute with an equal amount of water and allow to stand in a graduate for 2 hours.
4. Draw off the upper one-third of the liquid and replace with warm tap water.
5. Repeat this process until the supernatant fluid is clear (6-8 times).
6. Pour off the clear supernate and place the residue in a Petri dish to dry.
7. Transfer to a beaker, add 4 volumes of ether, and extract for 2 hours in a 37° C. water bath. Pour off the ether and repeat the procedure for a total of 3 extractions.
8. Remove the ether; dry in *vacuo* for 48 hours over sulfuric acid.

9. Pulverize the dry residue in a clean dry mortar.
10. Store in sterile ampules.
11. Make up a 1:100 dilution (by weight) of the dry powder in neutral isotonic saline. Allow to stand 4 hours at room temperature. Adjust the pH to 7.0. Continue the extraction in the refrigerator for 15-18 hours. Centrifuge for one-half hour at highest available speed. Pour off the supernate. Incubate in a 58° C. water bath for 1 hour. Centrifuge at high speed for one-half hour. Discard the sediment. Adjust the pH to 7.0, if necessary.

12. Store the 1:100 stock solution in the deep freeze.

#### PROCEDURE AND INTERPRETATION:

1. Make a 1:5000 dilution from the 1:100 stock solution. Keep in refrigerator until used. Small quantities of the 1:5000 dilution may be preserved in sterile screw-cap bottles in the deep freeze for prolonged periods.

2. Inject 0.1 ml of the 1:5,000 dilution intradermally.
3. For interpretation, see table 1.

### Precipitin Test

#### PROCEDURE:

1. The 1:100 dilution of antigen as prepared for the intradermal test above is used for the test.
2. Set up tubes for the test as follows:

First six tubes:	0.2 ml of patient's serum in each tube.
Seventh tube:	0.2 ml of normal human serum.
Last three tubes:	0.2 ml of infected rabbit's serum in each tube.

3. The tubes are respectively carefully overlaid with the following solutions:

- a. 0.2 ml stock *Trichinella* antigen (1:100)
- b. 0.2 ml antigen, 1:200 saline dilution
- c. 0.2 ml antigen, 1:400
- d. 0.2 ml antigen, 1:800
- e. 0.2 ml antigen, 1:1,600
- f. 0.2 ml saline
- g. 0.2 ml antigen, 1:100
- h. 0.2 ml antigen, 1:100
- i. 0.2 ml antigen, 1:200
- j. 0.2 ml antigen, 1:400

4. Negative sera remain clear. Positive sera develop a white ring at the interphase, within 30 minutes, and the antigen usually becomes cloudy.

## ECHINOCOCCOSIS

### Intradermal Test

#### PREPARATION OF ANTIGEN:

1. Remove fluid from human or animal hydatid cyst with a sterile needle and syringe using sterile technique. Turbid or purulent fluid is unsatisfactory.
2. Filter.
3. Check for sterility by culturing a sample of the fluid.
4. Dispense in small quantities (1 ml) in small sterile screw cap bottles and place in the deep freeze. The material will retain its potency for at least 6 months.

#### PROCEDURE AND INTERPRETATION:

1. Inject 0.2 ml of the antigen intradermally in the forearm.
2. A positive test is indicated by the almost immediate appearance of a wheal. Note: See table 1.

### Complement-Fixation Test

#### PREPARATION OF ANTIGEN:

1. Obtain fresh, bacteriologically sterile, hydatid fluid by aspiration from in-

fected sheep or cattle. Turbid or purulent fluid is unsatisfactory.

2. Chill approximately one liter of the fluid in the refrigerator.
3. Acidify by the addition of 100 ml of 5 percent trichloracetic acid.
4. Place in the refrigerator overnight to accelerate flocculation.
5. Remove from the refrigerator and add approximately 1 ml of 5 percent trichloracetic acid to the clear supernatant fluid. If no additional precipitate forms, go on to the next step. If further precipitation occurs, add approximately 50 ml of 5 percent trichloracetic acid and place in the refrigerator. This process is repeated until no further precipitate forms after addition of 1 ml of 5 percent trichloracetic acid.
6. Separate the protein precipitate by centrifugation at the highest speed available. Pour off the supernatant fluid.
7. Wash the precipitate in approximately 20 volumes of distilled water, cen-

- trifuge at high speed, and pour off the supernatant fluid. Repeat for total of 3 washings.
8. Suspend the precipitate in approximately 50 ml of distilled water and add 10 percent sodium hydroxide, drop by drop, until practically all of the precipitate is in solution.
  9. Centrifuge at high speed to separate the insoluble residue. The supernatant fluid contains the antigen. This is separated from the insoluble residue and chilled in the refrigerator. Add sufficient 1 N glacial acetic acid to reprecipitate the protein and allow to stand in the refrigerator overnight. If no additional precipitate forms after the addition of a few drops of 1 N glacial acetic acid, the material is centrifuged and washed free of acid as in step 7 and evaporated in a drying oven at 37° C. or over calcium chloride.
  10. Grind the precipitate into a fine powder and store over calcium chloride ride in a desiccator. Approximately
- 100 mg of antigen may be obtained for each liter of hydatid fluid.
11. Stock antigen solution is prepared by making a 1:1,000 dilution (by weight) of the purified antigen in slightly alkalized physiological saline solution.
  12. The stock antigen solution is sterilized by Seitz filtration or by addition of 0.5 percent chloroform. This antigen is approximately 10 times as potent as unpurified hydatid cyst fluid.

#### PROCEDURE AND INTERPRETATION:

1. The stock antigen solution is diluted to a final concentration of 1:5,000. The test is performed in the same way as the Kolmer Wassermann, substituting the purified hydatid fluid antigen.
2. The antigen is sensitive, specific, is not anticomplementary and gives very few false positive tests.
3. With degenerated cysts and recurrent cases, the complement fixation test may be negative. However, the intradermal reaction will usually be positive in these cases.

## SCHISTOSOMIASIS

### Intradermal Test

#### PREPARATION OF ANTIGEN:

1. Adult worms of *Schistosoma mansoni* or *Schistosoma japonicum* are recovered from the liver and mesenteric and portal veins of experimentally infected hamsters.
2. The worms are washed several times with sterile isotonic saline and once with distilled water, then transferred to 10 ml ampules for desiccation.
3. The contents are quickly frozen in a mixture of dry ice and ethanol (minus 78° C.) and dehydrated under reduced pressure from the frozen state.

4. After 18 hours, the ampules are sealed under vacuum using a cross fire torch and kept in a deep freeze until sufficient material is obtained for antigen extraction.
5. A glass tissue grinder and a quantity of ether are chilled to minus 20° C. in a deep freeze. One hundred mg of desiccated schistosomes are transferred to the chilled grinder, 10 ml of cold anhydrous ether are added (anesthetic ether is unsatisfactory for this purpose). Mixture is ground for 10 minutes with the grinder cylinder immersed in a calcium chloride ice-water bath (minus 15°–18° C.). The motor is run at slow speed to minimize heating.

The suspension of ground worms is poured into a chilled glass centrifuge tube and placed in the refrigerator.

6. Five ml of cold ether are added to the small amount of worm residue in the grinder and grinding is resumed for 2 minutes. This second suspension is then combined with the first and centrifuged for 30 minutes at 850 G in a refrigerated ( $3^{\circ}$ - $6^{\circ}$  C.) angle centrifuge. Following centrifugation, the supernatant ether is immediately decanted and traces of ether vapor aspirated from the centrifuge tube and grinder through a Richards' filter pump.
7. The residue is returned quantitatively to the grinder using 10 ml of veronal bicarbonate buffered salt solution (VBS). The stock solution consists of 83.8 gm NaCl, 2.52 gm NaHCO<sub>3</sub>, 3.00 gm sodium 5, 5-diethyl barbiturate, and 4.60 gm 5, 5-diethyl barbituric acid in distilled water sufficient to make 2,000 ml. The acid is dissolved in 500 ml hot distilled water and added to the solution of the other reagents. The whole is then cooled to room temperature, and made up finally to 2,000 ml with distilled water. Before use, one volume of the stock solution is diluted by adding four volumes of distilled water; the pH of the diluted solution is 7.3-7.4.
8. The mixture is ground slowly for 10 minutes at room temperature. The resulting suspension is poured into a cellulose nitrate centrifuge tube. The small amount of worm residue remaining in the grinder is then suspended by grinding for two minutes with an additional 5 ml of VBS, and then combined with the bulk of suspension.
9. The pooled material is transferred to a refrigerator where extraction is allowed to proceed for 4 hours.

10. The suspension is then centrifuged for 30 minutes at 850 G in the refrigerated centrifuge. The opalescent supernate is removed carefully to a beaker. One ml volumes of the extract are dispensed into ampules, lyophilized, and hermetically sealed.
11. The intradermal antigen is made by first reconstituting the antigen in 1 ml of sterile distilled water and then making 1:10,000 dilution to which phenol is added as a preservative in the ratio of 0.5 ml of liquid phenol per 100 ml of diluted antigen.

#### PROCEDURE AND INTERPRETATION:

1. Approximately 0.02 ml of diluted antigen is injected intradermally in the right forearm and a similar amount of buffered saline to which 0.5 percent phenol has been added is injected intradermally in the left forearm as a control.
2. A positive test consists of a flare and wheal which is significantly larger than the control.
3. The test has been shown to be positive in 45.0 percent of a group of 276 Puerto Rican soldiers exposed to *S. Mansoni*. The complement-fixation test was positive in 43.5 percent of the group. Positive stools were demonstrated in 18.8 percent. The test was negative in all of 158 control individuals who had never been exposed to schistosomiasis. Of 53 soldiers with positive stools, 49 (92.5 percent) had both positive intradermal and complement-fixation tests. (Horstman, Chaffee, and Bauman, 1954). Since this is a group specific reaction, the results would be expected to be comparable in cases of *S. hematobium* and *S. japonicum* infection.

#### Complement-Fixation Test

An improved complement-fixation test using the same antigen as for the intradermal test

(*vide supra*) has been described by Chaffee, Bauman, and Shapilo, 1954; Diagnosis of schistosomiasis by complement-fixation, Am. J. Trop. Med. and Hyg. 3:905-913. The procedure for the test is similar to that of the Kolmer Wassermann. Positive reactions occur in over 90 percent of cases. False positive tests may occur with syphilis; serologic tests for

syphilis should be performed to aid in differentiation. With few exceptions, if the complement-fixation test is positive, the intradermal reaction will always be positive. There is indication that after elimination of schistosome infection, the complement-fixation test will become negative and the intradermal test will remain positive.

Table 1. Immunologic Tests for Parasitic Diseases

Test	Authors	Source of Antigen	Interpretation
<b>AMOEBIASIS</b> Complement-fixation.	Bozicevich et al. (1946) ---	Cultures of <i>E. histolytica</i> ---	<ol style="list-style-type: none"> <li>Positive in 86 percent of cases of extraintestinal amoebiasis.</li> <li>Positive in 15 percent of cases of intestinal amoebiasis only.</li> <li>Positive in 0.7 percent of cases that had equivocal or no evidence of amoebiasis (McDearman and Dunham, 1952).</li> <li>Becomes negative after elimination of parasites by specific therapy.</li> </ol>
<b>LEISHMANIASIS</b> <i>L. braziliensis</i> : Intradermal ---	Pessoa, Pestana (1940) ---	Phenolized saline suspension of 12 day culture of <i>L. braziliensis</i> on N.N.N. medium.	One-tenth ml intradermally causes erythematous wheal in positive cases in 48 hours. Positive test obtained in 92 percent of cases in which <i>L. braziliensis</i> was later demonstrated. (Lopez and Leander, 1945).
Complement-fixation.	DaCunha, Dias (1938) ---	Cultures of <i>L. braziliensis</i> ---	High correlation of positive results in clinically active cases.
<i>Leishmania donovani</i> : Aldehyde test ---	Napier -----	No antigen -----	This is a non-specific test for increased serum globulin. Positive in 82 percent of patients with Kala-azar. False positives occur in any condition in which serum globulin is elevated.
Antimony test --- Complement-fixation.	Chopra (1936) ----- Grevial, Sen Gupta and Napier (1939) Sen Gupta (1945).	No antigen ----- Acid fast bacilli -----	Positive in 88 percent of cases. Positive in 93 percent of cases. More sensitive and becomes positive earlier than the other serologic tests for Kala-azar. (Sen Gupta, 1944).

Table 1. Immunologic Tests for Parasitic Diseases—Continued

Test	Authors	Source of Antigen	Interpretation
<i>Leishmania tropica:</i>			
Intradermal	Dubousky (1941)-----	Cultures of <i>L. tropica</i> -----	Reported to be positive in high percentage of cases.
Complement-fixation.	Senekjie (1943)-----	Cultures of <i>L. tropica</i> -----	Reported to be positive in high percentage of cases.
Agglutination	Senekjie (1943)-----	Cultures of <i>L. tropica</i> -----	Reported to be positive in high percentage of cases.
<b>TRYPANOSOMIASIS</b>			
<i>Trypanosoma cruzi:</i>			
Intradermal	Mayer, Pifano (1941)-----	Cultures of <i>T. cruzi</i> -----	Intradermal injection of 0.1 ml produces a papule with a surrounding area of erythema within $\frac{1}{2}$ hour in positive individuals. The wheal is 2-4 cm in diameter in 24 hours and fades to original size in 5 days. The test is considered to be specific. It is negative in leishmaniasis and malaria.
Agglutination	Senekjie (1943)-----	Cultures of <i>T. cruzi</i> -----	The test is said to be specific for <i>T. cruzi</i> infection.
Complement-fixation.	Kelser (1936)-----	Cultures of <i>T. cruzi</i> -----	The test is probably positive in a high proportion of cases. False positive tests may occur in patients infected with species of <i>Leishmania</i> .
<b>ASCARIASIS</b>			
Intradermal-----	Ransom, Harrison and Couch (1924).	Body fluid of <i>A. lumbricoides</i> .	A few drops of body fluid antigen are placed on a scarified area on the skin. A positive test is indicated by the immediate appearance of an erythematous wheal at the site of application. Lymphatic extension and systemic symptoms may occur but are usually not serious. A positive test indicates an experience with <i>Ascaris</i> sometime in the past and does not necessarily mean recent infection.

Table 1. Immunologic Tests for Parasitic Diseases—Continued

Test	Authors	Source of Antigen	Interpretation
<b>FILARIASIS</b> Intradermal-----	Bozicevich, Hutter (1944)-----	<i>Dirofilaria immitis</i> (Dog heart worm).	One-hundredth ml of diluted antigen injected intradermally produces a wheal which is larger than the control in 15 minutes in positive cases. The test is probably positive in over 90 percent of active cases of infection due to <i>W. bancrofti</i> and in a high percentage of infections due to <i>O. volvulus</i> and <i>Loa loa</i> . The test may be of particular value in early infections due to <i>W. bancrofti</i> before the worms have become mature enough to produce larvae.
Complement-fixation.	Bozicevich et al. (1947)-----	<i>O. volvulus</i> -----	Has some diagnostic value.
<b>STRONGYLOIDIASIS</b> Intradermal-----	Brannon (1943)-----	Washed filariform larvae of <i>Strongyloides</i> .	One-tenth ml of resuspended powdered antigen intradermally produces an urticarial wheal in a few minutes in positive cases. Brannon and Faust (1949) demonstrated positive test in all of 25 persons with chronic strongyloidiasis. All controls were negative except for one with exfoliative dermatitis and one moribund patient.
Precipitin-----	Brannon (1943)-----	Washed filariform larvae of <i>Strongyloides</i> .	Results same as intradermal.
<b>TRICHINIASIS</b> Intradermal-----	Bachman (1928)----- Sawitz (1937).	Larvae freed by digestion of muscle from infected rats.	One-tenth ml of purified, pulverized, reconstituted, diluted antigen is injected intradermally in one forearm. Positive cases: A small wheal at site of injection surrounded by a erythematous area about 5 cm in diameter appears, reaches its maximum in about 10 minutes, and begins to fade in 15–20 minutes. Test becomes positive about 14 days after infection and remains positive for an indefinite period. A positive test does not necessarily indicate a recent infection. Some individuals may show a delayed reaction as early as 9 days after ingesting larvae. Occasional false positives and false negatives occur.

Table 1. Immunologic Tests for Parasitic Diseases—Continued

Test	Authors	Source of Antigen	Interpretation
Precipitin-----	Roth (1945, 1946)-----	Larvae freed by digestion of muscle from infected rats.	Living larvae are incubated with patient's serum. Bubbles and granules appear around the mouth of the larvae in 24 hours in positive sera. Test becomes positive as early as 10-20 days after onset of symptoms. The test is said to be a more reliable index of recent infection than the intradermal reaction. False positive tests occur in lymphomas and leukemias, periarthritis, infectious mononucleosis, and quinine or arsenical therapy are associated with false positive reactions. (Southam et al., 1950; Bassen et al., 1949).
Precipitin-----	Sawitz, (1937)-----	Larvae freed by digestion of muscle from infected rats.	The interpretation of the test is essentially the same as for the test of Roth described above. The technique of the test is described in the text.
Complement-fixation	Bachman (1929)-----	Larvae freed by digestion of muscle from infected rats.	In the series of Frisch et al., all of 248 trichinosis patients had a negative C.F.T. at the time of onset. Twelve weeks later slightly over one-third were positive. Titers were highest at the 3-week testing (tests were made at 3, 6, and 12 weeks). A positive test is indicative of recent infection; only 12 per cent of asymptomatic cases showed a positive test. False positive tests have been reported in infectious mononucleosis (Bassen et al., 1949).
<b>SCHISTOSOMIASIS</b>			
Intradermal-----	Fairley, Williams (1927)--- Taliaferro, Taliaferro (1931) Horstman et al. (1954)	1. Adult schistosomes from infected laboratory animals. 2. Schistosome cercariae. 3. Tissue from molluscs infected with schistosomes. S. mansoni adults----- S. mansoni cercariae-----	The antigen is group specific. Positive reactions in over 90 percent of chronic infections. False positive reactions are apparently rare.
Precipitin-----	Oliver Gonzalez, Pratt (1944)		Positive reactions were obtained in 93 per cent of patients infected with S. mansoni. No false positives were noted in the control group.

Table 1. Immunologic Tests for Parasitic Diseases—Continued

Test	Authors	Source of Antigen	Interpretation
Complement-fixation	Chaffee et al. (1954)-----	<i>S. mansoni</i> or <i>S. japonicum</i> adults from infected laboratory animals.	The test is most useful in early cases before ova can be demonstrated, in old chronic cases in which extensive fibrosis prevents the discharge of eggs, and in unisexual infections. Positive reactions occur in over 90 per cent of cases. False positives may occur with syphilis; serologic tests for syphilis should be performed to aid in differentiation.
<b>PARAGONIMIASIS</b>			
Complement-fixation.	Faust (1949)-----	Macerated <i>P. westermani</i> adults.	This test is of greatest value in abdominal type infection where ova cannot be recovered; C.F.T. is positive in most of these cases.
<b>CYSTICERCIASIS</b>			
Intradermal-----	Faust 1949)-----	Fluid from any species of cysticerci in domestic animals.	Procedure same as intradermal test for hydatid disease. Positive results probably indicate the presence of infection. Negative results have been recorded in heavy infections.
Precipitin-----	Faust (1949)-----	Fluid from any species of cysticerci in domestic animals.	Procedure same as precipitin test for hydatid disease. Positive results probably indicate the presence of infection. Negative results have been recorded in heavy infections.
<b>ECHINOCOCCOSIS</b>			
Intradermal-----	Casoni (1911)----- Dew et al. (1925) Dennis (1937)	Hydatid fluid-----	A positive test is indicated by the formation of a wheal almost immediately after intradermal injection of 0.2 ml of antigen. A positive immediate reaction is present in almost all cases. Positive skin reactions may be obtainable many years after successful operative removal of a cyst. The intradermal test is superior to the C.F.T. False positive tests are said not to occur. The C.F.T. is usually positive but it is commonly negative when the cyst is degenerated and in recurrent cases.
Complement-fixation.	Dennis (1937)-----	Hydatid fluid-----	Results closely parallel the C.F.T.
Precipitin-----	Faust (1949)-----	Hydatid fluid-----	



## PART FOUR

# ARTHROPODS

### Chapter 9

## ARTHROPOD PARASITES AND DISEASE VECTORS

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Because of the widespread distribution of arthropods, their close association with troops, the role which they play both as parasites and as disease vectors, and the numerous other ways in which they may affect health and morale, it may sometimes be necessary for laboratory personnel to be prepared to determine whether arthropods present in an area are likely to be of medical importance. Various arthropods are often referred to the laboratory for identification, and the technician should be able to recognize representative forms. The laboratory technician is not expected to know the various species by their scientific names, but if called upon to send in a representative sampling of mosquitoes, he should be able to send mosquitoes and not a

variety of small beetles, flies, midges, moths, etc. It is the purpose of this chapter to familiarize the technician with the more common arthropods of potential importance and to aid him in identifying the various groups. Methods of collecting, preserving, and shipping will also be discussed.

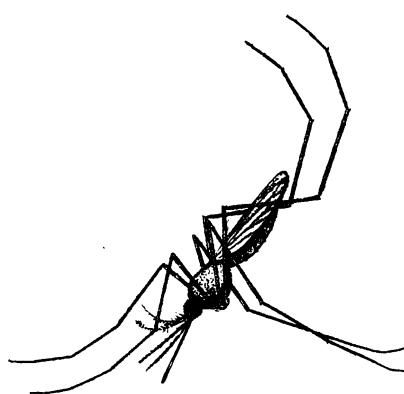
The close association which exists in nature between arthropods on the one hand, and the parasites of man on the other, should by this time be very firmly impressed upon the student of parasitology. A treatise on parasitology would be lacking in a most important area if some consideration were not given to arthropods in a separate chapter of the present manual.

### IMPORTANT ARTHROPOD VECTORS OF DISEASE

The discovery of the role which arthropods play in the dissemination of disease to the human population marked one of the greatest discoveries of all time. One needs only to consider the depredations of diseases such as plague, typhus, malaria, and yellow fever, and their effects on the history of mankind to begin to appreciate the tremendous importance of this group of animals. In spite of our present knowledge of the role they play in disease transmission, we still witness that more than one-half of the diseases which occur in the human population are transmitted by insects. Important arthropod-borne diseases are summarized in table 1.

#### Mosquitoes

From a standpoint of human health, mos-



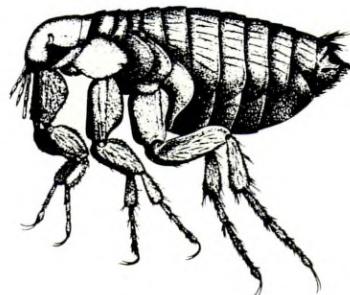
quitoes constitute the most important single group of vectors of disease to man. This is aside from the role which they play as intolerable pests in many parts of the world. Mosquitoes are known vectors of filariasis, dengue, yellow fever, the malarias, and a number of the virus encephalitides. The members of this group which are of prime medical importance fall into three genera; the *Anopheles*, *Aedes*, and *Culex*. *Anopheles* sp. derive their importance from the role which they play in malaria transmission; the *Aedes* sp. are associated with the transmission of dengue and yellow fever; *Culex* sp. are the principal vectors of the virus encephalitides. The genera mentioned above, and a number of others, are proven vectors of filariasis.

Mosquitoes breed in a wide range of aquatic environments, some genera and species displaying a high degree of selectivity, and others selecting much broader ranges of sites for deposition of their eggs. More unusual breeding places include the leaf axils of certain plants, crab holes, and sea water holding depressions in rocks along seacoasts. Less selective species oviposit in a wide range of ground water environments and man-made artificial containers. Other important biological variations in this group of insects are their blood-sucking habits, flight range, relative abundance at different seasons of the year, and degree of domesticity. The majority of mosquito species feed on plant juices and do not take a blood meal during their life cycle. Many species have a preference for the blood of warm-blooded animals other than man, and many are known to feed on cold-blooded animals. Some species range only a few hundred feet from their breeding sources; others are known to be capable of flight ranges as great as forty miles. Many breed in situations far removed from human habitation; others are very domestic, seeking breeding environments in close proximity to populated places. The biology of different species plays a vital part in determining their relative importance at different times and places. A knowledge of biological characteristics is the only sound

basis upon which control programs against pest mosquitoes and vectors of disease can be based.

### Fleas

Fleas come into close association with man primarily as ectoparasites of domestic animals and rodents. The species with which we are

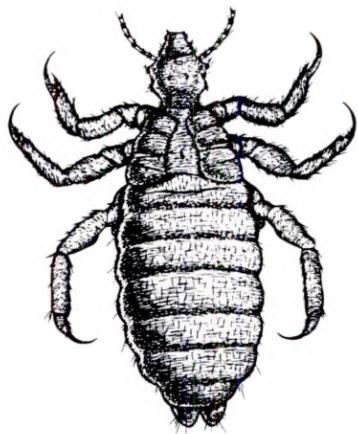


most concerned from a public health standpoint are those which infest rats, dogs, and cats. Only one species, *Pulex irritans* (the human flea) seeks a blood meal on man by preference. Fleas, in general, display a marked preference for the blood of certain warm-blooded animals, but in the absence of the host of choice they readily accept substitute hosts. Species infesting domestic animals are primarily pest species. Those infesting rodents can, under certain environmental conditions, transmit disease.

The diseases which fleas are capable of transmitting are primarily diseases of rodents in which man becomes an accidental intruder in a cycle which is normally perpetuated in the rodent population. Among these rodent diseases are plague, murine typhus, and tularemia. Fleas also serve as intermediate hosts for two species of tapeworms transmissible to man, namely *Hymenolepis diminuta* and *Dipyldium caninum*. The Chigoe flea, *Tunga penetrans* parasitizes many different species of warm-blooded animals including man. The immature female of this species actively burrows into the skin of the warm-blooded host to grow to maturity and produce eggs. The site of entry into the human skin is generally about the feet.

## Lice

The human head or body louse is worldwide in distribution and found wherever the level of sanitation of the population is low.



In modern wars, up to and including World War I, lice constituted one of the principal threats to the health and lives of troops in the field. The increased level of sanitation, together with the development of more effective insecticides, rendered this group of insects of very minor importance in World War II. Lice are true obligatory ectoparasites of man, spending their whole life cycle on the body or in the clothing of humans. Various factors influencing large segments of the population and contributing to the lowering of sanitation level favor the spread of lice. Thus, this pestilence becomes more prevalent during times of war, famine, and civil strife.

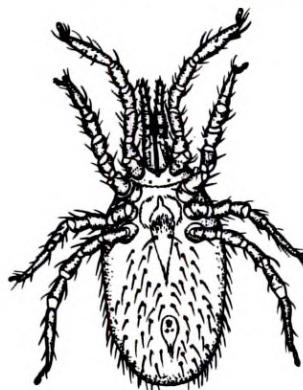
Diseases transmitted by lice include epidemic typhus, trench fever, and relapsing fever. In past history during pandemic periods, epidemic typhus spread rapidly over wide areas and millions of persons contracted the disease. Lice tend to leave the body of individuals who have fever and also to disperse quickly from the body of a dead person. These traits obviously favor the spread of louse-borne infections from one individual to another.

## Mites

Scrub typhus (Tsutsugamushi disease), rickettsialpox, and possibly epidemic hemorrhagic fever are transmitted by certain species of

larval mites. Scrub typhus assumed epidemic proportions among American troops in the field in certain portions of the Pacific area during World War II. This disease normally occurs in various species of ground rodents which serve as the reservoir for human infection. Mites feeding on infected rodents acquire the infection and eggs deposited by infected mites harbor the infectious organisms. This phenomenon is known as transovarian infection. Larval mites hatching from these infected eggs, and accidentally infesting humans, and feeding upon them, transmit the disease.

On the basis of epidemiological evidence, epidemic hemorrhagic fever may also be transmitted by larval mites. The peaks of high incidence for this disease in spring and fall correspond with the peaks of population of

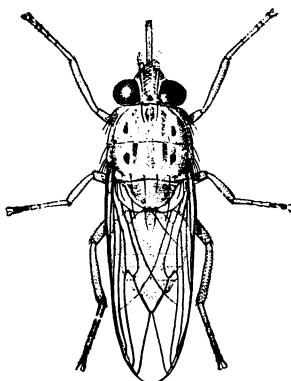


certain species of mites. In extensive field studies of this disease in Korea, where it was first encountered by American troops, the causative organism was never demonstrated. On purely epidemiological evidence, mite transmission of a virus is the most likely link in the cycle of human infection.

Various species of mites (chiggers) occur throughout the tropical and temperate regions of the world. Troops living in environments in which these mites occur, may, if unprotected, incur considerable annoyance and discomfort as a result of their bites.

## Tsetse Flies

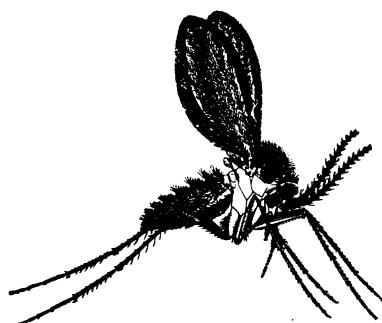
Several species of flies of the genus *Glossina* transmit African trypanosomiasis, a disease confined to tropical portions of the African



continent. The presence of this disease has undoubtedly been the greatest limiting factor in the economic development of this part of the world. Two morphologically identical species are recognized, each with a rather distinct geographical area of distribution and a recognizably different clinical picture. *Trypanosoma rhodesiense*, the causative organism of the milder form of African sleeping sickness, and the more virulent *Trypanosoma gambiense* are recognized. All untreated cases of infection with *Trypanosoma* sp. terminate in death, and populations have been so decimated in some parts of Africa that large areas are virtually uninhabited. The reservoir of human infection is certain species of large game animals, particularly of the antelope family.

#### Sandflies

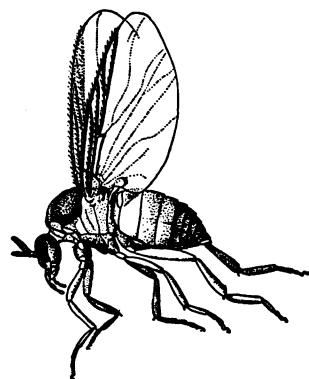
Members of this group belonging to the genus *Phlebotomus*, are implicated in the transmission of leishmaniasis, bartonellosis, and Papatas fever. There are three forms of



leishmaniasis, occurring over broad geographical areas, in different parts of the world. All are caused by morphologically indistinguishable organisms known collectively as Leishman-Donovan bodies. The three recognized forms of leishmaniasis are caused by *Leishmania infantum* (Oriental sore, Delhi boil), *Leishmania tropica* (Espundia), and *Leishmania donovani* (kala-azar). Espundia is widely distributed in southern Mexico, and Central and South America. Kala-azar is prevalent in India and adjoining regions, and Oriental sore occurs throughout areas adjoining the Mediterranean Sea. Dogs and cats are readily infected and serve as reservoirs of these diseases.

#### Black Flies

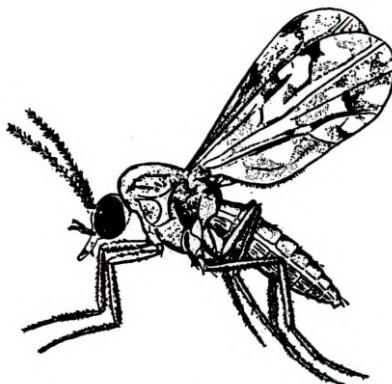
Black flies belonging to the genera *Eusimulium* and *Simulium* serve as intermediate hosts in the transmission of the filarial infection,



onchocerciasis, which occurs in Mexico, Central America, and parts of Africa. Of greater import, perhaps from a military standpoint, is the occurrence of tremendous numbers of these vicious biters in many regions in the north temperate zone. At some seasons of the year in Alaska, for example, their onslaughts are most annoying and troublesome to personnel working on aerial support activities out of doors. Immediately after the skin is punctured by these insects, blood flows from the wound inflicted. In some individuals, the immediate area surrounding the bite becomes swollen, and the swelling and attendant itching may persist for several weeks.

## Punkies or "No-See-Ums"

Punkies or "no-see-ums" are species of small vicious biting flies which are rather widely distributed throughout the world. They be-

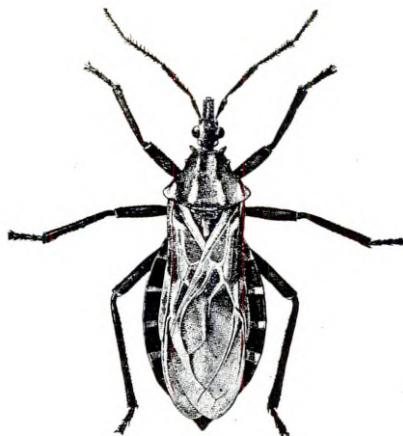


long to the genus *Culicoides* and, in addition to being severe pests in their own right, they serve as intermediate hosts for two species of filarial worms. In Africa, British Guiana, and New Guinea the filarial worm *Acanthocheilonema perstans* undergoes a portion of its cycle, and is transmitted by species of *Culicoides*. A filarial worm, *Mansonella ozzardi*, is transmitted by a species of black fly in the British West Indies.

These flies attack in the daytime, bite fiercely, and their bites may continue to be irritating for weeks. They attack principally at the neck, waist, and shoe line, often in large numbers; and the scratching of the sites of the bites results in exudative, serous sores which may become secondarily infected. Conspicuous red scars sometimes persist for many weeks.

## Assassin Bugs

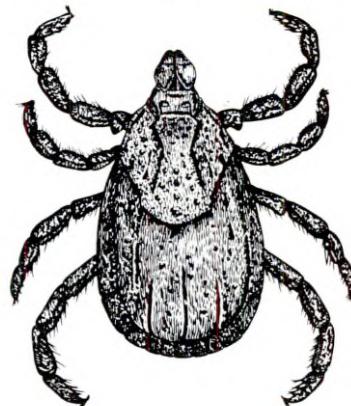
These bugs belong to the family *Reduviidae* and are known under various common names including "kissing bugs," "assassin bugs," and "Reduviids." Chagas' disease, widespread in Central and South America, and caused by a species of *Trypanosome*, is transmitted by a number of species of assassin bugs, notably *Triatoma* sp. The infection is not transmitted through the bite of the insect, but rather through contamination of the bite wound with the feces of the insect which contains the infective stage of the parasite. Opos-



sums and armadillos are readily fed upon by these bugs, and they serve as reservoirs of the disease in nature. Naturally infected armadillos and opossums, as well as naturally infected assassin bugs, have frequently been encountered in various places in southeastern United States, but thus far, human infection has not been observed in this country.

## The Hard Ticks

The hard ticks have a dorsal shield, which on the male covers the entire dorsal surface, but which on the female covers only the an-



terior third to one-half of the body. Both male and female hard ticks are blood suckers. They normally remain attached to the host for a considerable length of time, taking only one blood meal in each of the stages, larval, nymphal, and adult. When not on the host, hard ticks are usually found on the ground, infesting bushes and shrubs.

In the United States, the two most important hard ticks which serve as vectors of

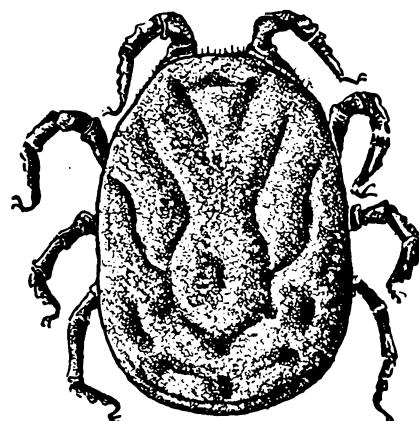
disease are *Dermacentor andersoni* and *D. variabilis*. These two species can transmit Rocky Mountain spotted fever, Q fever, various forms of encephalitis, and tularemia. Ticks are also implicated as vectors of a number of other less important viral and rickettsial diseases.

*Dermacentor andersoni*, and possibly *D. variabilis*, can cause a condition known as tick paralysis. Tick paralysis is not an actual disease, but a series of symptoms caused by a toxic substance secreted by the salivary gland. This toxic material, and the resulting paralysis, can result only from the bite of the female tick and is apparently associated with the production of eggs. Although paralysis may occur when the tick attaches in any location, it is most commonly associated with the presence of the tick at the back of the neck or along the spinal column. This paralysis sometimes resembles poliomyelitis and incorrect diagnoses are sometimes made. Complete removal of the tick usually results in rapid termination of symptoms, but if respiratory paralysis has already become manifest before the tick is removed, the patient may not recover.

### The Soft Ticks

Soft ticks belong to the family *Argasidae*, characterized by the fact that the dorsal sur-

face lacks a shield, thus giving the body the appearance of a sack when viewed from above. These ticks are primarily ectoparasites of



birds though they may be found on bats and cattle as well as man. Those species which frequently serve as disease vectors to man commonly live in the house with man. This is especially true in areas where mud, adobe, or thatch is used as building material and where floors are of dirt.

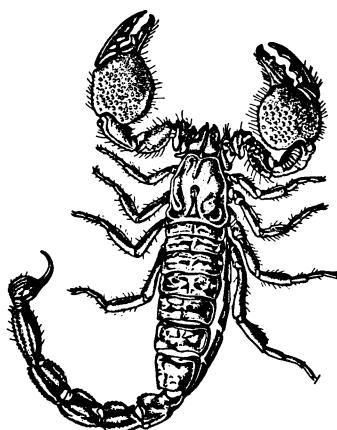
Ticks of the genus *Ornithodoros* transmit various forms of relapsing fever in many parts of the world. One of the most serious of these is African relapsing fever. Members of this genus also transmit European relapsing fever, as well as a form of relapsing fever which occurs in southern United States and Mexico.

## VENOMOUS ARTHROPODS

The sting of certain species of scorpions and centipedes and the bite of the black widow spider may be quite dangerous. The reaction to these stings and bites will vary with the amount of venom injected, the site of injection, and the age of the individual afflicted. While healthy adults generally recover, the aged and the very young may die if proper care is not immediately instituted.

### Scorpions

Scorpions are long Arachnids which give the appearance of having five pairs of legs. The claws, however, are not legs. The abdomen is composed of thirteen segments, the



first seven of which might be termed as being

part of the body, while the last six form the "tail." The last segment is modified in the form of a hooked stinger which carries two venom sacs. Scorpions feed mainly on insects and other small forms of animal life, grasping and crushing their prey with the claws. If the prey is too large or too active to be crushed easily, the stinger is brought into use. The tail is carried arched over the back and the stinger is inserted by a forward and downward thrust. Because of its many-jointed structure, the tail can be twisted so that the stinger can be used in almost any direction. The venom produces paralysis in the victim which can then be eaten more easily.

Many fatally poisonous species of scorpions occur throughout the world. Of the many species which occur in the United States, only two are known to be capable of causing human fatalities. Man is stung by scorpions most frequently while walking about barefoot at night or when putting on shoes in which scorpions have hidden. Man may also be stung when handling or cleaning up piles of trash and thus disturbing scorpions which may be hiding under such materials. Poisonous species occur principally throughout the warmer regions of the world and are especially prevalent in the Mediterranean region, particularly the North African coast.

### Centipedes

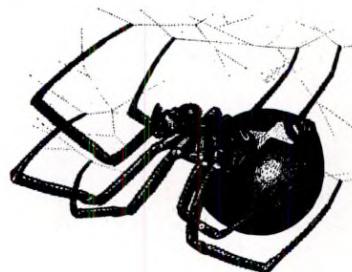
Centipedes belong to the class *Chilopoda*,



which are characterized by the possession of one pair of legs on each body segment. The first pair of appendages are modified by poison claws. The injection of venom through these poison claws enables centipedes to immediately paralyze or kill their prey (insects and other small animals). While all centipedes possess these claws, only a few species are large enough, and have jaws which are sufficiently strong, to penetrate the skin of man. Two American species attain a length of 4–6 inches at maturity, and they are capable of inflicting very painful bites. The largest species attain a length of 10 inches and are reputedly very poisonous. Persons are usually bitten in bed or when putting on items of clothing in which centipedes have hidden during the night.

### Black Widow Spiders

The female black widow spider is 12–14 mm in length while the male is about half that size. In the species which occurs in the United



States, the entire body is usually a bright, shiny black except for the markings. Black widow spiders found in various parts of the world vary from the American species in marking. For example, an important species found in the Mediterranean region is jet black throughout and bears no characteristic color markings. In all *Latrodectus* sp. there are short, black hairs covering the body and legs but they are so fine that they are not usually noticed. In the North American species the female has, on the undersurface of the abdomen, an hourglass-shaped spot which is usually bright red, but orange to cream colors may occur on those found in some geographical regions.

The bite of the female black widow spider, although not as dangerous as generally believed, can produce death. The death rate is claimed to be between 1 and 5 percent according to different authors. Although very young children or very aged individuals are more

susceptible to black widow spider venom, most of the deaths actually occur among males bitten in the genital area, usually while using outdoor privies. In the United States the majority of deaths are found among migrant workers in the California vegetable fields.

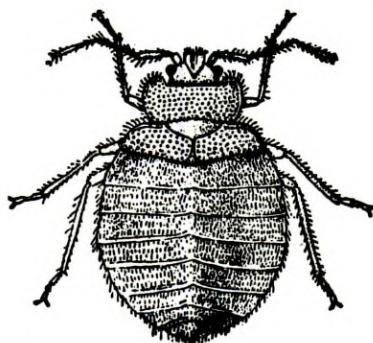
## TRUE ECTOPARASITES

This discussion is limited to arthropods which live upon, or in close association with man, throughout their life cycle and derive their nourishment primarily from humans. So far as is known, they are not associated in any manner with the transmission of disease.

### Bedbugs

Bedbugs have never been proven to transmit any disease to man naturally, although the bite of the insect produces intense itching which may persist for days or weeks in some individuals. Some persons may show allergic reactions to the bites. Since bedbugs live in such close association with man, it is fortunate that they do not transmit disease. The fact that they do not is probably due to their structure and habits; they are unable to regurgitate blood which they have ingested, and they do not defecate at the time of biting. Experimentally, they have been proven capable of transmitting plague, salmonellosis, tularemia, and anthrax.

Bedbugs live in cracks and crevices in walls, baseboards, and flooring, or in the small tight areas in and on beds. They also frequently live in the rolled edges of mattresses, and eggs may be laid in any of these areas. Bedbugs are

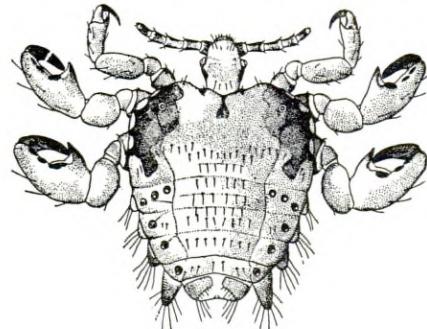


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easily transported in clothing and baggage, and they will readily move from one house to another by way of walls, pipes, and electrical conduits. Infestations are frequently encountered in military installations, particularly overseas bases in countries with low standards of sanitation.

### Pubic Louse

The crab or pubic louse is usually found on the hairs of the genital region but may occur on other parts of the body, particularly in



heavy infestations. It is readily recognized by the following characteristics: its area of attachment; its stout legs which terminate in chitinous, recurved, grasping claws; and the square outline of the body.

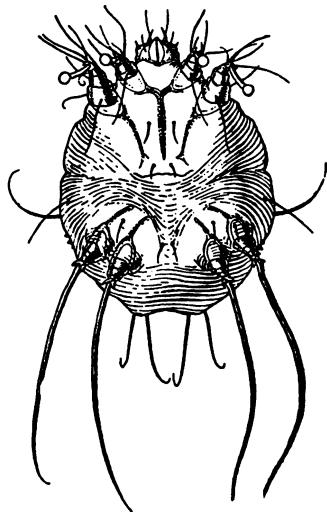
The pubic louse has never been incriminated in disease transmission. However, infestations cause considerable discomfort, and some people are very sensitive to the bites, which may produce macular swellings and a great deal of pruritus. In persons with infestations of long duration, the skin may become thickened and show spots of hyperpigmentation which persist for long periods of time after the condition has been successfully treated.

Transmission is by contact with infested individuals, their clothing or bedding, or other

objects with which they have come in contact, particularly toilet seats. When an infestation is discovered in a military population, many individuals will usually be found to be infested, and a general campaign of treatment of all persons living in close contact is indicated. Military populations into which new individuals are constantly entering, are particularly vulnerable to infestation.

### Itch Mites

The itch mite, *Sarcoptes scabiei*, is worldwide in distribution and completely democratic in its choice of victims. It is a parasite of both man and domestic animals, the disease which they cause being known as scabies in



man and mange in domestic animals. When adults from an infected man or animal gain access to the skin of a susceptible, the adult mite burrows into the skin, digging a burrow in the upper layers of the epidermis. Eggs are deposited in the burrows and hatch in a few days. The liberated larvae dig new burrows and mature in a short period of time. The favorite sites of infestation are the interdigital spaces between the fingers and the groin. The activities of the mites cause intense itching and discomfort and within a relatively short interval of time, large areas of skin may become involved. Scratching may lead to breaks in the skin which serve as avenues of entry for secondary invaders. Untreated infections may be of long duration giving rise to the classical "seven-year-itch."

When infection by itch mites is suspected, examine the hands of the patient carefully under strong light, observing for short tortuous tracts which terminate in raised whitish papules. Wetting the skin with a small amount of glycerin will make these tracts and papules stand out more distinctly. With a sterile pin, carefully dissect open the papule. The small, gleaming white adults can be seen to migrate out of the broken skin over the papule. Skin scrapings from suspected infected areas can also be made, mounted on glass slides with 20 percent sodium hydroxide, and examined under low power for the presence of adults and eggs.

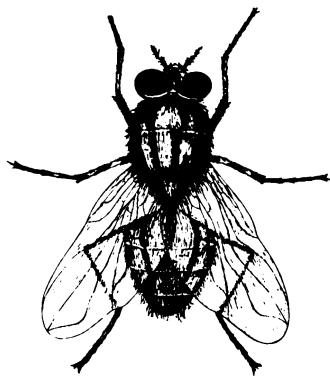
## ARTHROPODS AS MECHANICAL CARRIERS OF DISEASE

On the basis of their ability to transmit disease, arthropods can be classified into two broad categories—those which serve as biological carriers and those which carry the etiological agents of disease without playing an essential part in the cycle proper. The principal arthropods which act as mechanical carriers are houseflies and cockroaches.

### Houseflies

The housefly has followed man to every geographical area which he has inhabited. The seasons in which they pose a menace are delineated by temperature and moisture; and

when these conditions are favorable, flies usually abound. When the principal limiting

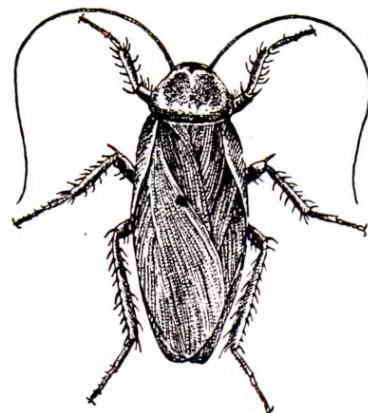


factor, namely food, is also in abundance fly populations are generally excessive. Their breeding habits and preference for various foods, including excrement of warm-blooded animals, are well known. Considering their habits, it is little wonder that there is positive laboratory proof for their transmission of at least thirty different diseases. Cholera, typhoid, amebic and bacillary dysentery, various diarrheas, tetanus, anthrax, trachoma, yaws, leprosy, tuberculosis, and certain helminth infections (by eggs) are among the diseases listed.

In certain studies in the United States over 98 percent of flies captured within dwellings were found to be of the species *Musca domestica*, which is commonly known as the housefly. It is characterized principally by the occurrence of four longitudinal black stripes which extend the length of the dorsal side of the thorax.

### Cockroaches

Cockroaches are cosmopolitan in distribution, there being four species which infest human



habitation. Different species abound either together or separately in different regions of the world. These insects frequent filthy situations, readily feeding on both human excreta and sputum. Their favorite haunts are the places where food is stored, prepared, or eaten. Their habits afford abundant opportunity to carry the etiologic agents of diseases on their hairy bodies and antennae. Viable *cholera vibrio* have been found in cockroaches over three days after being fed to cockroaches, thus their excreta as well as their vomitus may also carry infectious organisms.

## MYIASIS

Invasion of body tissues by the immature stages of various species of flies is termed myiasis. In a large majority of these infections, man is an accidental intruder in a cycle a portion of which is normally spent either in animals other than man or in decomposing organic matter. Myiasis can be conveniently divided into three types on the basis of the degree of adaptation of the various species of flies involved to the parasitic habit. These types include *specific*, *semi-specific*, and *accidental* myiasis. Specific myiasis is caused by obligatory parasites, well adapted to the parasitic habit, which require a warm-blooded animal for completion of their life cycle. Semi-specific myiasis is caused by flies, the larvae of which are semi-obligatory parasites capable of development either in a warm-blooded host or in decomposing organic matter. Accidental myiasis is caused by flies, the larvae of which

are non-obligatory or facultative parasites, poorly adapted to the parasitic habit, and which normally develop in non-living organic matter.

Myiasis-producing larvae most generally gain entrance to the body either through natural apertures or through broken or injured the body include the conjunctiva of the eyes, skin. Natural openings facilitating entry into the ears, the external nares, the mouth, and the external orifices of the genito-urinary and gastro intestinal tract. Infection may occur as a result of deposition of ova by oviparous species of flies, deposition of larvae of viviparous species, or through the migration of larvae from other habitats to sites of entry. Ova or larvae may also be accidentally ingested with contaminated food or drink.

The medical importance of the various myiasis is most closely associated with the species

of fly involved, the site attacked, the intensity of the infection, and the accessibility of the involved areas for effective removal or destruction of the organism. Inflammation and pain are usually present. Localization in the nasopharyngeal region by the more invasive species may result in erosion of soft tissues, cartilage, and bone with resultant impairment of function, disfigurement, and death. Secondary infections accompanying larval activity may give rise to festering purulent wounds and generalized septicemia with death ensuing. In untreated cases with extensive involvement of deeper inaccessible body tissues, prognosis is poor.

Bats, screwworm flies, and flesh flies are among the most important myiasis-producing species. From a parasitological standpoint, the taxonomy of these arthropods is of secondary importance. Even experts find the task of identifying flies from the larval forms difficult. Whenever viable larvae believed to be dipterous are recovered in laboratory examinations,

a portion should be preserved by killing with hot (not boiling) 70 percent alcohol and tubing in a suitable tight container with 70 percent alcohol added as preservative. If only dead maggots are obtained, they should be similarly preserved. Live maggots should be placed in a dish containing raw meat in a glass jar, the bottom of which is filled with loose moistened sand to a depth of about one inch. The container should be covered with loose cotton or securely fastened gauze. Depending on their stage of development, the maggots will generally penetrate into the meat and remain there until they are ready for pupation. The larvae eventually will migrate to the soil to pupate and after several days emerge as adult flies. The jar should be periodically examined for emerged adults. Both larvae and adults should be processed according to instructions given elsewhere in this chapter and forwarded to an experienced entomologist for identification.

Table 1. Myiasis in Man

## Specific Myiasis

COMMON NAME OF SPECIES	GROUP OR SPECIES NAME	HOST RELATIONSHIP	REMARKS	KNOWN GEOGRAPHICAL DISTRIBUTION
Congo floor maggots.	<i>Auchmeromyia luteola.</i>	Sucks blood at night only.	Larvae hide in ground during daytime.	Tropical Africa.
Tumbu flies-----	<i>Cordylobia anthropophaga</i>	Young larvae invade unbroken skin.	Larvae normally leave body after 8-9 days.	Tropical Africa.
Human botflies-----	<i>Dermatobia hominis.</i>	Larvae invade exposed skin areas.	Fly glues eggs to mosquito or other "carrier" insect.	Tropical South America, Central America, and Mexico.
Horse botflies-----	<i>Gasterophilus</i> spp-----	Larvae wander beneath skin.	Three species are normal parasites in gut of horse.	World-wide.
Cattle botflies-----	<i>Hypoderma</i> spp-----	Larvae wander beneath skin.	Normal parasites beneath skin on backs of cattle.	World-wide.
Sheep botflies-----	<i>Oestrus ovis</i> -----	Larvae invade nose, pharynx, conjunctival sac.	Normal parasites in naso-pharynx of sheep.	World-wide.
Head botflies of horses.	<i>Rhinoestrus purpureus.</i>	Larvae invade naso-pharynx and conjunctival sac.	Normal parasites in head passages of horses.	Southern and Eastern Europe, N. Africa, Asia Minor.
Primary screw-worm flies.	<i>Callitroga americana.</i>	Larvae invade nose, ears, sinuses, and wounds (rarely unbroken skin).	Mortality, 8 percent-----	Warmer regions of the Western Hemisphere.
Old World screw-worm flies.	<i>Chrysomyia bezziana.</i>	Larvae invade nostrils, ears, conjunctiva, vagina, or open wounds.	Larvae complete development and drop out in 7-14 days.	Oriental and Ethiopian Regions.
Flesh flies-----	<i>Wohlfahrtia magnifica.</i> <i>W. vigil</i> -----  <i>W. meigenii</i> -----	Invade nose, ears, wounds of all types. Invade unbroken skin.  Invade unbroken skin.	May cause death-----  Parasitize babies, especially in neck region.  Parasitize babies, especially in neck region.	Mediterranean, Near East, and Russia. Nearctic Region.  Western U.S.A.

## Semi-Specific Myiasis

Common screw-worm flies.	<i>Callitroga macellaria.</i>	Invade open wounds---	Normal in decaying flesh.	Widespread in Western Hemisphere.
Green bottle flies.	<i>Lucilia</i> spp-----	Usually attack only diseased tissue.	Formerly used in surgery and treatment of osteomyelitis	World-wide.

Table 1. Myiasis in Man—Continued

COMMON NAME OF SPECIES	GROUP OR SPECIES NAME	HOST RELATIONSHIP	REMARKS	KNOWN GEOGRAPHICAL DISTRIBUTION
Blue bottle flies.	<i>Calliphora</i> spp. <i>Cynomyia</i> spp.	Invade wounds, cutaneous ulcers, and malodorous apertures.	Usually attack only diseased tissue.	, World-wide.
Black bottle flies.	<i>Phormia</i> spp.	Invade wounds, cutaneous ulcers, and malodorous apertures.	Formerly used in treatment of osteomyelitis.	World-wide.
Flesh flies-----	<i>Sarcophaga</i> spp-----	Invade wounds, cutaneous ulcers, and malodorous apertures; may penetrate unbroken skin.	Many species are viviparous.	World-wide.
Stable flies-----	<i>Stomoxys calcitrans</i> .	Invades open wounds-----	Rather rare in man-----	World-wide.
Houseflies-----	<i>Musca</i> spp-----	Invade open wounds-----	Presumed to be a secondary invader.	World-wide.

## Accidental Myiasis—Intestinal

Houseflies-----	<i>Musca domestica</i>	Inhabit various portions of the intestinal tract.	Infection acquired by ingestion of eggs or larvae or by flies depositing eggs or larvae on anus during defecation.	More or less generally distributed throughout the world.
Green bottle flies.	<i>Lucilia</i> spp.			
Blue bottle flies.	<i>Calliphora</i> spp.			
Flesh flies-----	Family Sarco-phagidae.			
Latrine flies-----	<i>Fannia scalaris</i> .			
Cheese skippers.	<i>Piophila casei</i> .			
Rat tail maggots	<i>Tubifera</i> spp.			

## Accidental Myiasis—Genito-Urinary

Houseflies-----	<i>Musca domestica</i> ---	Urinary tract, including bladder; genital passages of females.	Infection acquired by migration of larvae from intestinal tract or by flies depositing eggs or larvae on genital aperture, especially of females.	Not common but occurs widely the world over.
Cheese skippers.	<i>Piophila casei</i> .			
Rat tail maggots	<i>Tubifera</i> spp.			
Latrine flies-----	<i>Fannia</i> spp.			
Green bottle flies	<i>Lucilia</i> spp.			
Blue bottle flies.	<i>Calliphora</i> spp.			
Flesh flies-----	<i>Sarcophaga</i> spp.			

Table 2. Summary of the More Important Arthropod Vectors of Human Disease

Exclusive of Myiasis

VECTOR	DISEASE	SPECIFIC AGENT
Millipedes----- Copepoda (water fleas)-----	Hymenolepiasis----- Diphyllobothriasis----- Dracontiasis----- Gnathostomiasis----- Sparganosis-----	<i>Hymenolepis diminuta.</i> <i>Diphyllobothrium latum.</i> <i>Dracunculus medinensis.</i> <i>Gnathostoma spinigerum.</i> <i>Diphyllobothrium spp.</i>
Decapoda (crayfish, crabs, etc.)-----	Paragonimiasis-----	<i>Paragonimus westermani.</i>
Mites-----	Bertelliasis----- Rickettsialpox----- Tsutsugamushi-----	<i>Bertiella studeri.</i> <i>Rickettsia akari.</i> <i>Rickettsia tsutsugamushi.</i>
Ticks-----	Boutonneuse fever----- Bullis fever----- Colorado tick fever----- Encephalitis, Russian spring-summer----- Louping ill----- Maculatum disease----- Plague----- Q Fever----- Relapsing fever (endemic)----- Spotted fever----- Tularemia-----	<i>Rickettsia conora.</i> <i>Rickettsia sp.</i> Filtrable virus. <i>Erro sylvestris virus.</i> Virus sp. <i>Rickettsia sp.</i> <i>Pasteurella pestis.</i> <i>Coxiella burnetii.</i> <i>Borrelia duttoni.</i> <i>Rickettsia rickettsiae.</i> <i>Pasteurella tularensis.</i>
Black flies-----	Onchocerciasis----- Tularemia-----	<i>Onchocerca volvulus.</i> <i>Pasteurella tularensis.</i>
Cockroaches-----	Amebiasis----- Balantidiasis----- Cholera----- Dysentery, bacillary----- Food poisoning----- Giardiasis----- Gongylonemiasis----- Hymenolepiasis----- Poliomyelitis----- Paratyphoid fever----- Typhoid fever-----	<i>Endamoeba histolytica.</i> <i>Balantidium coli.</i> <i>Vibrio comma (cholera).</i> <i>Shigella spp.</i> <i>Staphylococcus sp., Salmonella sp., Streptococcus sp.</i> <i>Giardia lamblia.</i> <i>Gongylonema pulchrum.</i> <i>Hymenolepis diminuta.</i> Virus types 1, 2, 3. <i>Salmonella spp.</i> <i>Salmonella typhosa.</i>
Cone-nose bugs-----	Trypanosomiasis (Chagas)-----	<i>Trypanosoma cruzi.</i>
Deer flies-----	Loaiasis----- Tularemia-----	<i>Loa loa.</i> <i>Pasteurella tularensis.</i>
Eye gnats-----	Conjunctivitis----- Tropical ulcer----- Trachoma----- Yaws-----	Bacteria spp. Mixed bacteria and spirochete flora. Virus. <i>Treponema pertenue.</i>

Table 2. Summary of the More Important Arthropod Vectors of Human Disease—Continued

VECTOR	DISEASE	SPECIFIC AGENT
Fleas.....	Dipylidiasis..... Hymenolepiasis..... Plague..... Typhus fever (endemic).....	<i>Dipylidium caninum.</i> <i>Hymenolepis diminuta.</i> <i>Pasteurella pestis.</i> <i>Rickettsia typhi.</i>
Housefly and other filth flies.....	Amebiasis..... Bacillary Dysentery..... Balantidiasis..... Cholera..... Cysticercosis..... Food poisoning.....  Giardiasis..... Paratyphoid fever..... Poliomyelitis..... Trachoma..... Trichuriasis..... Tropical ulcer..... Typhoid fever..... Yaws.....	<i>Endamoeba histolytica.</i> <i>Shigella</i> spp. <i>Balantidium coli.</i> <i>Vibrio comma</i> (cholera). <i>Taenia solium.</i> <i>Staphylococcus</i> sp. <i>Salmonella</i> sp., <i>Streptococcus</i> sp. <i>Giardia lamblia.</i> <i>Salmonella</i> sp. Virus types 1, 2, 3. Virus. <i>Trichuris trichiura.</i> Mixed bacteria and spirochete flora. <i>Salmonella typhi.</i> <i>Treponema pertenue.</i>
Lice.....	Relapsing fever (epidemic)..... Trench fever..... Typhus fever (epidemic).....	<i>Borrelia recurrentis.</i> <i>Rickettsia quintana.</i> <i>Rickettsia prowazekii.</i>
Mosquitoes.....	Dengue..... Encephalitis (California; Equine, Eastern, Venezuelan, Western; Japanese B; Russian, spring-summer; West Nile). Filariasis, Bancroft's..... Filariasis, Malayan..... Malaria..... Rift Valley fever..... Tularemia..... Yellow fever.....	<i>Charon</i> sp. virus. Virus.  <i>Wuchereria bancrofti.</i> <i>W. malayi.</i> <i>Plasmodium</i> spp. Virus. <i>Pasteurella tularensis.</i> <i>Charon evagatus.</i>
Moths and beetles.....	Hymenolepiasis.....	<i>Hymenolepis diminuta.</i>
Biting gnats.....	Acanthocheilonemiasis..... Mansonelliasis..... Microfilaria streptocerca.....	<i>Acanthocheilonema perstans.</i> <i>Mansonella ozzardi.</i> <i>Acanthocheilonema streptocerca.</i>
Sand flies.....	Bartonellosis..... Espundia..... Kala-azar..... Oriental sore..... Sandfly fever.....	<i>Bartonella bacilliformis.</i> <i>Leishmania brasiliensis.</i> <i>L. donovani.</i> <i>L. tropica.</i> <i>Pappataci</i> virus.
Tsetse flies.....	Trypanosomiasis.....	<i>Trypanosoma gambiense</i> , <i>T. rhodesiense</i> .

**Table 3. Summary of the More Important Human Diseases Transmitted by Arthropods**

Bacterial Diseases

KNOWN AS	VECTOR, CARRIER, OR ESSENTIAL IN LIFE CYCLE (GROUP)	SPECIES	KNOWN REGION OF DISTRIBUTION
Cholera.....	Nonbiting flies and cockroaches.	<i>Musca</i> spp. and domestic cockroaches.	World-wide.
Conjunctivitis.....	Nonbiting flies.....	<i>Hemophilus</i> spp..... <i>Hippelates collusor</i> ..... <i>H. flavigens</i> ..... Note: Undoubtedly other nonbiting Diptera the world over.	United States. California. Coastal U.S.A. in Gulf region.
Dysentery.....	Nonbiting flies and other arthropods living in close contact with humans.	Examples: <i>Musca</i> spp. and members of the family Blattidae.	World-wide.
Plague.....	Fleas.....	<i>Citellophilus tesquorum</i> ..... <i>Chiastopsyllus rossi</i> ..... <i>Diamanus montanus</i> ..... <i>Dinopsyllus lypusus</i> ..... <i>Hoplopsyllus anomalus</i> ..... <i>Nosopsyllus fasciatus</i> ..... <i>Oropsylla silantievi</i> ..... <i>Pulex irritans</i> ..... <i>Rhopalopsyllus cunicola</i> ..... <i>Synosternus pallidus</i> ..... <i>Xenopsylla astia</i> ..... <i>X. brasiliensis</i> ..... <i>X. cheopis</i> ..... <i>X. eridox</i> ..... <i>X. nubicus</i> .....	Mongolia. South Africa. California and Oregon. South Africa. California and Oregon. Temperate zone. Mongolia. World-wide. Argentina and Ecuador. West Africa. India, Ceylon, Burma, Mesopotamia, Mombasa. Belgian Congo, Kenya, Uganda, Bechuanaland. World-wide. South Africa. Tropical East and West Africa.
Tularemia.....	Deer flies, ticks, and mosquitoes.	<i>Amblyoma americanum</i> ..... <i>Dermacentor andersoni</i> ..... <i>D. variabilis</i> ..... <i>D. silvarum</i> ..... <i>Aedes cinereus</i> ..... <i>Culex apicalis</i> ..... <i>Chrysops discalis</i> .....	United States. United States. United States. U.S.S.R. Sweden. U.S.S.R. Western U.S.A.
Typhoid and Paratyphoid Fever.	Flies and cockroaches....	<i>Musca</i> spp. and other filth flies and cockroaches.	World-wide.

Helminths

Bertielliasis.....	Mites.....	<i>Orbatid</i> mites.....	Africa, Asia, Mauritius, West Indies.
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Table 3. Summary of the More Important Human Diseases Transmitted by Anthropods—Continued

KNOWN AS	VECTOR, CARRIER, OR ESSENTIAL IN LIFE CYCLE (GROUP)	SPECIES	KNOWN REGION OF DISTRIBUTION
Cysticercosis (bladder worm).	Nonbiting flies-----	<i>Musca domestica</i> and other filth flies.	World-wide.
Diphyllobothriasis (fish tapeworm).	Copepods containing infective stage.	<i>Cyclops</i> spp----- <i>Diaptomus</i> spp.	Europe and North America, Japan, and scattered areas of Africa.
Dipylidiasis (dog tape-worm).	Fleas or lice containing infective stage.	<i>Ctenocephalides canis</i> C. <i>felis</i> <i>Pulex irritans</i> <i>Trichodectes canis</i>	World-wide
Hymenolepiasis diminuta (rat tapeworm disease).	Arthropods containing infective stage.	<i>Coleoptera</i> ----- <i>Dermaptera</i> <i>Diplopoda</i> <i>Lepidoptera</i> <i>Orthoptera</i> <i>Siphonaptera</i>	India, Russia, Japan, Italy, U.S.A.
Hymenolepiasis nana (dwarf tapeworm).	Beetles and fleas containing infective stage.	<i>Siphonaptera</i> ----- <i>Coleoptera</i>	World-wide.
Trichuriasis (whipworm)-----	Nonbiting flies-----	<i>Musca</i> spp. and other filth flies.	World-wide.
Acanthocheilonemiasis-----	Biting gnats-----	<i>Culicoides austeni</i> C. <i>grahami</i> .	Rain forests of West and Central Africa, tropical South America and New Guinea, and coastally in Trinidad and Panama.
Bancroft's filariasis-----	Mosquitoes-----	<i>Culex quinquefasciatus</i> ----- <i>Culex pipiens</i> ----- <i>Aedes aegypti</i> ----- <i>Anopheles albimanus</i> ----- <i>Anopheles darlingi</i> ----- <i>A. gambiae</i> ----- <i>A. punctulatus</i> ----- <i>Aedes polynesiensis</i> ----- Note: Also 60 additional species of mosquitoes.	World-wide. Central China, Japan, and Egypt. West Africa, Belgian Congo, New South Wales, Surinam, West Indies. Caribbean area. Brazil. West Africa, Belgian Congo, Zanzibar. New Guinea, various South Pacific islands. Numerous Polynesian islands.
Dracontiasis (guinea worm).	Copepods-----	<i>Cyclops</i> spp-----	Africa, India, Russia, Middle East.
Gongylonemiasis-----	Cockroaches-----	<i>Blattidae</i> -----	Italy, Bulgaria, U.S., Russia, Ceylon, China.
Loaiasis (eye worm)-----	Deer flies-----	<i>Chrysops dimidiata</i> , <i>C. distinctipennis</i> , <i>C. silacea</i> .	Tropical Africa, especially the Congo River basin.

Table 3. Summary of the More Important Human Diseases Transmitted by Anthropods—Continued

KNOWN AS	VECTOR, CARRIER, OR ESSENTIAL IN LIFE CYCLE (GROUP)	SPECIES	KNOWN REGION OF DISTRIBUTION
Malayan filariasis-----	Mosquitoes-----	<i>Anopheles barbirostris</i> ----- <i>A. hyrcanus complex</i> ----- <i>A. umbrosus</i> ----- <i>Mansonia annulata</i> ----- <i>M. annulifera</i> ----- <i>M. Longipalpis</i> ----- <i>M. indiana</i> ----- <i>M. uniformis</i> -----	Indonesia, Malaya, and China. Indonesia, Malaya, and China. Malaya. Indonesia, Malaya, and Philippines. Indonesia, Malaya, India, and Ceylon. Malaya. Malaya. Malaya.
Mansoneliasis (Ozzard's filariasis).	Biting gnats-----	<i>Culicoides furens</i> -----	West Indies, Central and South America.
Onchocerciasis (blinding filariasis).	Black flies-----	<i>Simulium callidum</i> ----- <i>S. metallicum</i> ----- <i>S. ochraceum</i> ----- <i>S. damnosum</i> ----- <i>S. neavei</i> -----	Guatemala, Southern Mexico. Guatemala, Southern Mexico. Guatemala, Southern Mexico. Tropical Africa. Tropical Africa.
Paragonimiasis (lung flukes).	Infective stage in crabs and crayfish.	<i>Astacus japonicus</i> ----- <i>A. similis</i> ----- <i>Eliocheir sinensis</i> ----- <i>Potoman dehaani</i> ----- <i>P. denticulatus</i> ----- <i>Pseudothlphusa iturbei</i> -----	Orient. Orient. China. Africa, China, South America. Orient. Venezuela.
<b>Protozoa</b>			
Amebiasis-----	Nonbiting flies-----	<i>Musca</i> spp. and other filth flies and cockroaches.	World-wide.
Balantidiasis-----	Nonbiting flies-----	<i>Musca</i> spp. and other filth flies.	World-wide.
Giardiasis-----	Nonbiting flies-----	<i>Musca</i> spp. and other filth flies.	World-wide.
Espundia (muco-cutaneous leishmaniasis).	Sandflies-----	<i>Phlebotomus</i> spp-----	Central and South America.
Kala-azar (visceral leishmaniasis).	Sandflies-----	<i>Phlebotomus argentipes</i> ----- <i>P. chinensis</i> ----- <i>P. intermedius</i> ----- <i>P. longipalpis</i> ----- <i>P. major</i> ----- <i>P. perniciosis</i> ----- <i>P. sergenti mongolensis</i> -----	India. China. South America. Brazil. Greece. Italy, Sicily. China.

Table 3. Summary of the More Important Human Diseases Transmitted by Anthropods—Continued

KNOWN AS	VECTOR, CARRIER, OR ESSENTIAL IN LIFE CYCLE (GROUP)	SPECIES	KNOWN REGION OF DISTRIBUTION
Oriental sore (cutaneous leishmaniasis).	Sandflies.....	<i>Phlebotomus papatasii</i> ..... <i>Phlebotomus</i> spp.....	India. Africa, Asia.
Malaria.....	Mosquitoes.....	Numerous sp. of mosquitoes of the genus <i>Anopheles</i> .	64° North to 32° South in tropical and temperate zones except certain islands.
African Trypanosomiasis.....	Testse flies.....	<i>Glossina palpalis</i> ..... <i>G. tachinoides</i> ..... <i>G. morsitans</i> ..... <i>G. swynnertoni</i> .....	Equatorial Africa. West Africa. East Africa. Tanganyika.
South American Trypanosomiasis.	Assassin bugs.....	<i>Panstrongylus</i> spp..... <i>Rhodnius</i> spp..... <i>Triatoma</i> spp..... Note: Also other genera and species	Panama, South America. South America, Mexico. U.S.A., South America, Mexico, and Central America.

**Rickettsia**

Typhus, endemic.....	Fleas.....	<i>Ctenocephalides felis</i> ..... <i>Nosopsyllus fasciatus</i> ..... <i>Xenopsylla cheopis</i> ..... <i>X. astia</i> .....	Texas. Europe, North America. World-wide. Orient.
Typhus, epidemic.....	Lice.....	<i>Pediculus humanus</i> .....	World-wide.
Trench fever.....	Lice.....	<i>Pediculus humanus</i> .....	Western Ukraine.
Rickettsialpox.....	Mites.....	<i>Allodermanyssus sanguineus</i> .....	North Atlantic Seaboard, U.S.A.
Typhus, scrub (Tsutsugamushi).	Mites.....	<i>Trombicula akamushi</i> , <i>T. deliensis</i> , and <i>T. fugi</i> .	Japan, Australasian region, East Indies, and the Philippines.
Boutonneuse fever.....	Ticks.....	<i>Amblyoma hebraeum</i> , <i>Haemaphysalis leachi</i> , <i>Hyalomma rufipes</i> , <i>Rhipicephalus appendiculatus</i> , <i>R. simus</i> . <i>Rhipicephalus sanguineus</i> ..... <i>Ixodes holocyclus</i> ..... <i>Dermacentor nuttalli</i> , <i>D. silvarum</i> and <i>Haemaphysalis concinna</i> .	East, Central and South America. Mediterranean Basin, the Crimea and India. North and South Queensland. Russia and Eastern and Central Siberia.
Bullus fever (Lone Star fever).	Ticks.....	<i>Amblyoma americanum</i> .....	Texas.

**Table 3. Summary of the More Important Human Diseases Transmitted by Anthropods—Continued**

KNOWN AS	VECTOR, CARRIER, OR ESSENTIAL IN LIFE CYCLE (GROUP)	SPECIES	KNOWN REGION OF DISTRIBUTION
Spotted fever-----	Ticks-----	<i>Amblyoma americanum</i> ----- <i>Dermacentor andersoni</i> ----- <i>D. variabilis</i> -----  <i>Amblyoma brasiliensis</i> , <i>A. cajennense</i> , <i>A. striatum</i> . <i>Rhipicephalus sanguineus</i> ---	Oklahoma and Texas. Western United States. Eastern and Southern United States. Panama, Colombia, and Brazil. Mexico.
Maculatum disease-----	Ticks-----	<i>Amblyoma maculatum</i> -----	South Central and South-eastern United States.
Q fever-----	Ticks-----	Seventeen tick species have thus far been found naturally infected.	World-wide.

**Central Nervous System Viral Infections**

California virus-----	Mosquitoes-----	<i>Aedes dorsalis</i> and <i>Culex tarsalis</i> .	San Joaquin Valley, California.
Eastern equine encephalitis.	Mosquitoes, mites, and lice.	<i>Culiseta melanura</i> ----- <i>Mansonia perturbans</i> ----- <i>Dermyassus gallinae</i> and <i>Eomenacanthus stramineus</i> .	Louisiana, U.S.A. Georgia, U.S.A. Tennessee, U.S.A.
Western equine encephalomyelitis.	Mosquitoes and mites-----	<i>Culex tarsalis</i> , <i>Culex</i> spp., <i>Aedes dorsalis</i> , <i>Culiseta inornata</i> , <i>Anopheles freeborni</i> , <i>Dermyassus gallinae</i> , <i>D. americanus</i> , <i>Liponyssus bursa</i> , and <i>L. sylviarum</i> .	Mid-western and Western, U.S.A.
Venezuelan equine encephalitis.	Mosquitoes-----	<i>Aedes taeniorhynchus</i> ----- <i>Mansonia titillans</i> ----- <i>Anopheles neomaculipalpis</i> -----	South America. Trinidad, B.W.I. South America.
Japanese B encephalitis.	Mosquitoes-----	<i>Aedes chemulpoensis</i> -----  <i>Culex tritaeniorhynchus</i> , <i>C. fatigans</i> , and <i>C. annulirostris</i> . <i>Culex pipiens</i> , var. <i>pallens</i> -----	North China.  U.S.S.R. and much of the Far East.  Sino-Japanese areas, Southwest Pacific Islands and Maritime Provinces of Siberia.
Russian spring-summer encephalitis.	Ticks-----	<i>Haemaphysalis concinna</i> -----  <i>H. japonica</i> <i>Dermacentor</i> ----- <i>Ixodes persulcatus</i> -----	European U.S.S.R., Siberia, Far Eastern Maritime Provinces.  Eastern Maritime Provinces. U.S.S.R.

**Table 3. Summary of the More Important Human Diseases Transmitted by Anthropods—Continued**

KNOWN AS	VECTOR, CARRIER, OR ESSENTIAL IN LIFE CYCLE (GROUP)	SPECIES	KNOWN REGION OF DISTRIBUTION
St. Louis encephalitis-----	Mites and mosquitoes-----	<i>Dermanyssus gallinae</i> , <i>Culex tarsalis</i> , <i>C. pipiens</i> , <i>C. quinquefasciatus</i> , and <i>Aedes dorsalis</i> .	U.S.A., principally Midwest.
Semliki Forest encephalitis.	Mosquitoes-----	<i>Aedes abnormalis</i> -----	Uganda.
West Nile encephalitis-----	Mosquitoes-----	<i>Culex antennatus</i> , <i>C. pipiens</i> , and <i>C. univittatus</i> .	Tropical Africa.
Poliomyelitis-----	Nonbiting flies and cockroaches.	<i>Musca</i> spp., other nonbiting flies and <i>Blattidae</i> .	World-wide.

**Spirochetes**

Endemic relapsing fever-----	Ticks-----	<i>Ornithodoros asperus</i> ----- <i>O. crossi</i> ----- <i>O. erraticus</i> ----- <i>O. hermsi</i> ----- <i>O. marocanus</i> ----- <i>O. moubata</i> , and <i>O. savignyi</i> ----- <i>O. parkeri</i> ----- <i>O. rufid</i> ----- <i>O. talaje</i> ----- <i>O. tartakovskyi</i> ----- <i>O. tholozani</i> ----- <i>O. turicata</i> ----- <i>O. verrucosus</i> -----	Asia. Northern India. Portugal, Spain, Tunisia. Western U.S.A., British Columbia. Morocco, Spain. Africa. Western U.S.A.. Central and South America, Mexico. Central and South America. U.S.S.R. Iran, Iraq, Syria, Israel, Cyprus, and U.S.S.R. Mexico, Western U.S.A. Iran.
Epidemic relapsing fever-----	Body louse-----	<i>Pediculus humanus</i> -----	World-wide.
Tropical ulcer-----	Nonbiting flies-----	<i>Musca</i> spp----- <i>Sarcophaga</i> spp. and other filth flies.	Cosmopolitan in tropical regions of the world.
Yaws-----	Flies-----	<i>Hippelates flavipes</i> ----- <i>Musca domestica</i> complex-----	Caribbean Region. Africa and subtropical regions.

## **COLLECTION, PRESERVATION, AND SHIPMENT OF ARTHROPODS**

Specimens most generally handled by laboratory technicians are those brought into the laboratory by Army personnel and their dependents. It is usually possible to determine the general group to which such organisms belong, i.e., spiders, ticks, centipedes, scorpions, etc. When this is not possible, it may be necessary to process these specimens by preserving and shipping them to service installations staffed with experts in arthropod identification.

In certain situations, particularly in more remote areas, it may be necessary to collect arthropods of potential medical importance, and forward these to appropriate laboratories for identification. Arthropods which may be collected include, among others, flies, mosquitoes, fleas, mites, and ticks.

### **Collection of Arthropods**

The items of equipment most commonly utilized in collecting arthropods are light traps, killing bottles, nets, suction bottles, and small camel's-hair brushes. Light traps are useful for obtaining a sampling of the population of flying insects, particularly mosquitoes. In order to obtain adequate sampling and to assure that specimens will be obtained in good condition, several rules should be observed. Light traps should be so located that they give a sampling of the population which may be coming into contact with military personnel. They must be attended to on a regular schedule daily, operated during the hours of darkness, and the insects which have been caught should be removed from the traps early each morning. The contents of the catching jar should be spread out on white paper and the insects carefully sorted and counted. With a little experience, insects which are being sampled can be easily separated from the numerous other species attracted to light traps.

Killing bottles are useful for the collection of insects resting on surfaces, or insects alighting on their hosts for the purpose of obtaining a blood meal. Killing bottles should be of fairly heavy glass, and of a size suited to the particular purpose for which they will be used.

Small ones may be made from heavy, pyrex test tubes, while wide mouth, cork-capped bottles are suitable for preparing larger tubes. The killing agent is usually calcium, potassium, or sodium cyanide. The granular or flake cyanide is placed in a cloth bag or nested in the bottom of the container in cellucotton. The cyanide is then covered with a cellucotton plug consisting of several layers, or with about one-half inch of sawdust. A thick mixture of freshly prepared plaster of Paris and water should then be poured over material to a depth of about one-half inch. The container should then be placed in the open air, in a safe place and allowed to dry for several hours. It is then corked and kept covered at all times when not in use. An effective and safer catching tube can be made by filling the bottom of a suitable container to a depth of about 2 inches with finely cut rubber bands, and covering with several tight fitting discs of blotting paper. Five to ten milliliters of chloroform are added to the tube, and if kept corked, the tube will remain effective for several weeks. When the concentration of chloroform no longer gives adequate knock-down, the killing agent should be replenished. Various items of collecting and catching equipment are illustrated in figures 9-1 and 9-2.

It may sometimes be desirable to collect larval specimens, particularly mosquitoes. They can be collected from their aquatic habitat with dippers, transferred to clean water with medicine droppers, and brought into the laboratory for processing.

### **Preservation of Arthropods**

Soft-bodied forms such as Dipterous and mosquito larvae and smaller adult arthropods (fleas, lice, etc.) are most conveniently preserved by immersion in 70 percent alcohol, equal parts of 70 percent alcohol and glycerin, or 2 percent formalin. Live arthropods may be placed directly into the preservative, but better specimens can usually be obtained by killing them in hot, but not boiling, water.

In preserving mosquito larvae, it is preferable to kill them in Peterson's KAAD solution.

The KAAD solution contains kerosene (1 part), isopropyl alcohol (8 parts), glacial acetic acid (1 part), and dioxan (1 part). Place the larvae in a small dish and with a pipette, withdraw as much of the water as possible. Cover the specimens with KAAD mixture and allow them to remain in the solution overnight. With a pipette, withdraw as

much of the solution as possible, rinse the specimens with two or three changes of 70 percent alcohol, and preserve them in 70 percent alcohol or other suitable preservative.

A convenient method of preserving mosquito larvae and small arthropods for storage or shipment is to place them in discarded dental procaine hydrochloride tubes (see fig. 9-2D).

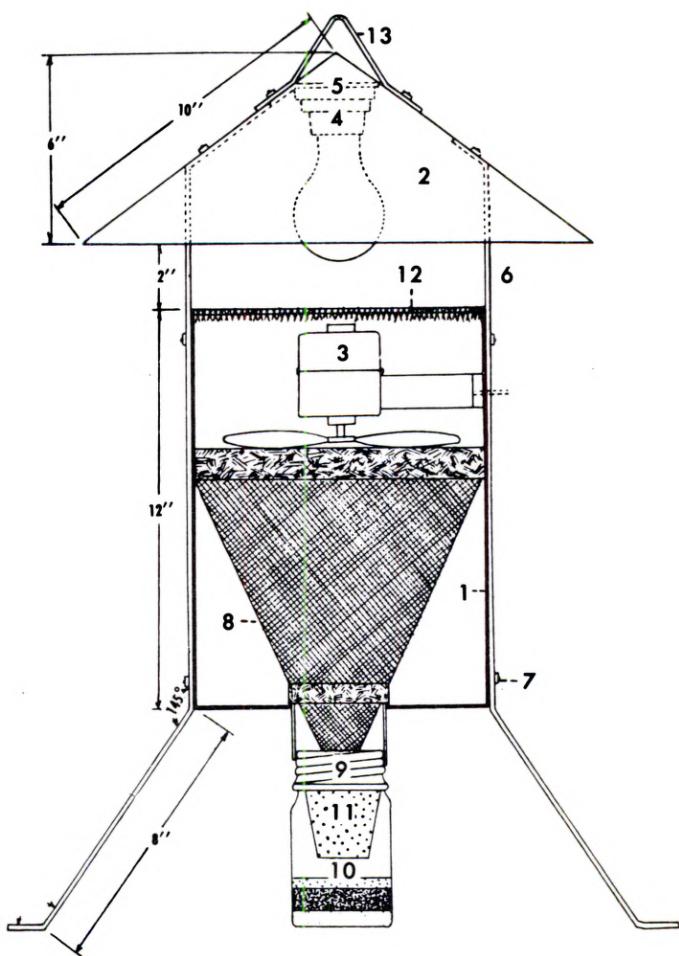
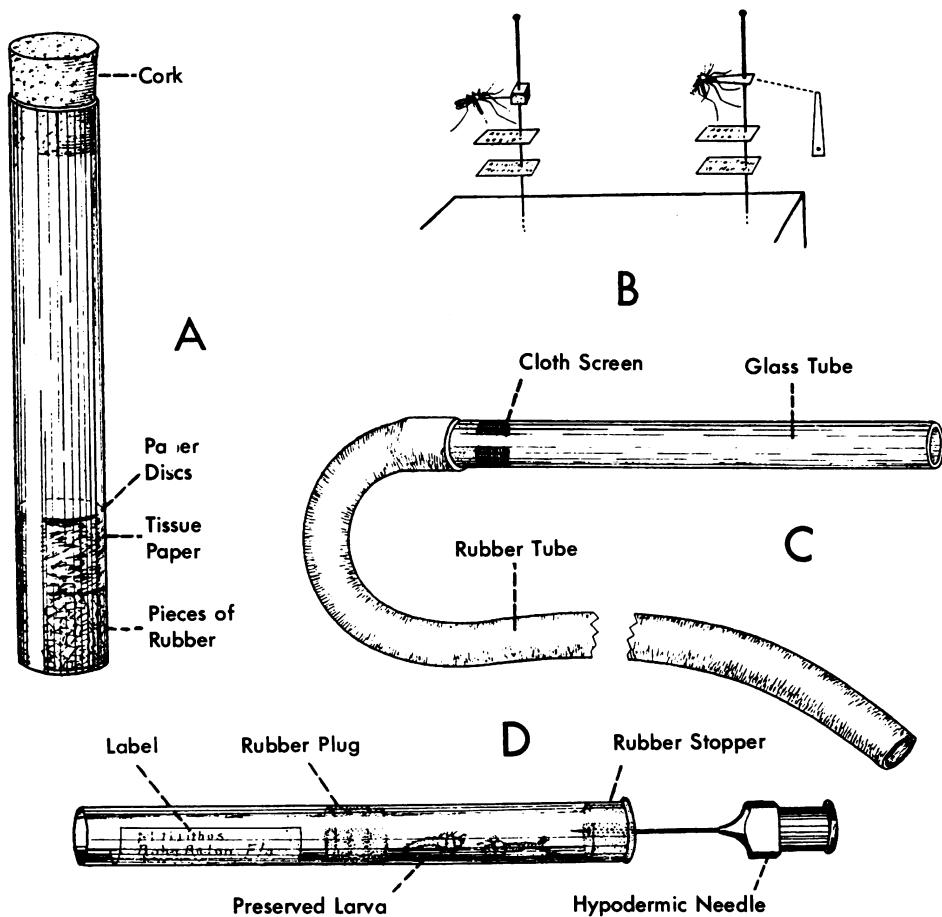


Figure 9-1. Diagram of the New Jersey Mosquito Trap (adapted from Mulherm, 1942).

- 1, Galvanized iron cylinder, 22 gauge, 9 inches inside diameter (cut away to show interior).
- 2, Roof of galvanized iron.
- 3, Motor and 8-inch fan.
- 4, Porcelain receptacle for light bulb.
- 5, Sponge rubber to absorb vibration.
- 6, Three supporting ribs of band iron ( $\frac{1}{8}$ " by 1").
- 7, Screws and nuts for assembling trap.
- 8, Screen funnel of 16 mesh bronze wire.
- 9, Mason jar cap.
- 10, Jar with cyanide for killing insects.
- 11, Perforated paper cup.
- 12, Galvanized screen  $\frac{3}{8}$ " mesh.
- 13, Loop for carrying or hanging trap.



**Figure 9-2.** A, Chloroform tube for killing adult insects. B, adult insects mounted on micropin and triangular paper point. C, Aspirator for collecting adult mosquitoes. D, Tube for preserving and storing arthropods.

A small quantity of the fluid preservative is first introduced into the tube, specimens are then transferred to the tube and a hypodermic needle is inserted through the rubber cork on one end. The rubber plug is then pressed downward with a blunt object until all of the entrapped air has been pushed out through the needle, and the needle is removed. This procedure stores specimens in convenient sized tubes and enables removal of entrapped air bubbles which unduly agitate stored material. It also provides on the end opposite of the specimen tube a convenient place in which to store record data. Larger specimens which are best preserved in a fluid preservative may be shipped in screw-cap vials of appropriate size.

Adult specimens of insects which must be kept dry are most conveniently transferred to

ointment boxes for storage or shipment. The specimens should be cushioned in the boxes by first cutting out two circular pieces of absorbent cotton and two circular pieces of lens paper. A circle of cotton is placed in the bottom of the ointment box and overlaid with a circle of lens paper. The specimens are then placed in the box, covered with a circle of lens paper upon which is placed the other circular cotton pad.

#### Shipment of Arthropods

Do not send living specimens through the mails. In shipping specimens in glass containers, special care must be taken in packing to prevent breakage. In the tropics, particular care should be taken to store arthropods in dry containers. Mold and insect pests will soon destroy dry arthropod specimens if they are

not properly cared for. Ointment tins or pill boxes containing specimens should be enclosed in mailing tubes or other sturdy containers for shipping. If pinned adults are sent, the pins must be forced firmly into place and the mounting box must be enclosed within another sturdy shipping box, and suspended within by a cush-

ioning layer of cotton, excelsior or the like. All shipped material should be accompanied by complete data as to locality, date, collector's name, and other pertinent information as to the requirement for identification and the individual to whom this information should be returned.



## Appendix 1

# STAINS AND SOLUTIONS

### **Acid Alcohol (1)**

Alcohol, ethyl, 70 percent.....	99 ml
Hydrochloric acid (HCl), concentrated ..	1 ml

### **Acid Fuchsin-Fast Green Stain for Intestinal Protozoa (2) (Lawless)**

Acetone ( $\text{CH}_3\cdot\text{CO}\cdot\text{CH}_3$ ).....	50.0 ml
Glacial acetic acid ( $\text{CH}_3\text{COOH}$ ).....	50.0 ml
Formaldehyde, USP (HCHO).....	10.0 ml
Schaudinn's solution.....	890.0 ml
Acid fuchsin.....	2.5 gm
Fast green FCF.....	1.0 gm

Mix the four solutions and add the two dyes. Mix very thoroughly until the dyes are dissolved. Store the prepared stain in a tightly stoppered brown bottle for several months prior to use. After this period of ripening, the staining solution remains stable and can be used indefinitely.

### **Alcohol Eosin (3)**

Aqueous eosin Y, 3 percent.....	100 ml
Alcohol, ethyl ( $\text{C}_2\text{H}_5\text{OH}$ ), absolute.....	125 ml
Distilled water.....	375 ml

A few drops of glacial acetic acid may be added if desired.

### **Alcohol-Formalin-Acetic Acid Fixative (4)**

Formalin (HCHO), commercial.....	100 ml
Alcohol, ethyl ( $\text{C}_2\text{H}_5\text{OH}$ ), 95 percent.....	250 ml
Glacial acetic acid ( $\text{CH}_3\text{COOH}$ ).....	50 ml
Glycerin ( $\text{C}_3\text{H}_8(\text{OH})_3$ ).....	100 ml
Distilled water.....	500 ml

### **Alcohol, Formula for Dilution From 95 Percent Alcohol (5)**

EXAMPLE: How much 95 percent alcohol should be q.s. to 100 ml to make up 100 ml of 85 percent alcohol?

$85:95::x:100$  or 8,500 divided by 95 equals 89.4 ml of alcohol to be q.s. to 100 ml.

EXAMPLE: How much 95 percent alcohol should be q.s. to 100 ml to make up 100 ml of 70 percent alcohol?

$70:95::x:100$  or 7,000 divided by 95 equals 72.6 ml of alcohol to be q.s. to 100 ml.

Statement of equation: Percent alcohol desired is to 95 percent alcohol as the number of ml of 95 percent alcohol to be used is to 100 ml.

### **Alsever's Solution (6)**

Dextrose ( $\text{C}_6\text{H}_{12}\text{O}_6$ ).....	2.050 gm
Sodium citrate ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ ).....	0.800 gm
Sodium Chloride (NaCl).....	0.420 gm
Citric acid ( $\text{C}_6\text{H}_8\text{O}_7$ ).....	0.055 gm
Distilled water.....	100.000 ml

### **Alum Cochineal (7)**

Aluminum potassium sulphate $(\text{AIK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O})$ .....	30 gm
Cochineal.....	30 gm
Distilled water.....	400 ml

Mix the salt, dye, and distilled water and boil for 1 hour. Cool to room temperature and filter, retaining the filtrate. Boil the filter paper containing the precipitate for 30 minutes. Filter again and add this filtrate to the first filtrate and boil for 30 minutes. Filter again and q.s. the filtrate to 400 ml.

### **Antibiotic Solution (Penicillin and Streptomycin) (8)**

Dissolve 100,000 units of penicillin and 100,000 units of streptomycin in 20 ml of sterile distilled water. Store in refrigerator in glass stoppered bottle. This solution contains 5,000 units of penicillin and 5,000 units of streptomycin per ml.

### **Anticoagulant, Sodium Citrate (9)**

Sodium citrate ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ ).....	2 gm
Physiological saline.....	100 ml

Mix ingredients thoroughly. To citrate blood add 1 ml of the above solution to each 5 ml of blood to be citrated.

### **Anticoagulant, Heparin (10)**

Heparin <sup>1</sup> .....	10.00 mg
Physiological saline <sup>1</sup> .....	0.25 ml

<sup>1</sup> Quantities required for each tube.

Transfer saline solution to test tube or centrifuge tube and add powdered heparin. Mix thoroughly and place in a 37° C. incubator until saline has evaporated. Each tube contains sufficient anticoagulant to heparinize 5 ml of blood. To heparinize several tubes, make up sufficient quantity containing 10 mg heparin per 0.25 ml (40 mg per ml) and pipette 0.25 ml of this solution into each tube.

### **Beemer's Stain for Vital Staining of Culture Stages of Trypanosomes and Leishmania (CDC modification) (11)**

Sodium citrate ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ ).....	1. 10 gm
Sodium chloride ( $\text{NaCl}$ ).....	0.65 gm
Mercuric chloride ( $\text{HgCl}_2$ ), saturated solution in water.....	0.10 ml
Brilliant cresyl blue.....	1. 10 gm
Methylene blue, saturated aqueous solution.....	0.50 ml

Place a few drops of material from culture on a slide and coverslip. Place a drop of the vital stain on one margin of the coverslip and allow it to run under. Progressive slowing down and staining of the organisms will be observed.

### **Bohmer's Hematoxylin (12)**

<i>Solution A</i>	
Hematoxylin crystals.....	1 gm
Alcohol, ethyl, absolute ( $\text{C}_2\text{H}_5\text{OH}$ ).....	12 ml
<i>Solution B</i>	
Alum (aluminum potassium sulfate) ( $\text{AIK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ ).....	1 gm
Distilled water.....	240 ml

Solution A should be aged until dark for best results. Just prior to use, 2 or 3 drops of Solution A are added to each 5 ml of Solution B.

### **Borax Carmine (13)**

Carmine.....	3 gm
Borax ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ).....	4 gm
Distilled water.....	100 ml
Alcohol, ethyl, 70 percent.....	100 ml

### **Bouin's Fixative (14)**

Pieric acid ( $\text{C}_6\text{H}_2(\text{NO}_2)_3\text{OH}$ ), saturated solution in distilled water.....	75 ml
Formalin ( $\text{HCHO}$ ), 40 percent solution..	25 ml
Glacial acetic acid ( $\text{CH}_3\text{COOH}$ ) .....	5 ml

Mix ingredients and store in hard glass bottle.

### **Buffer Solution for Balamuth's Egg Infusion Medium (15)**

#### *1 M Potassium dibasic phosphate (Solution A)*

Potassium dibasic phosphate ( $\text{K}_2\text{HPO}_4$ ) .....	87.09 gm
Distilled water....q.s. to.....	500.00 ml

Place the phosphate salt in a 500 ml volumetric flask, dilute with a portion of distilled water and dilute to the mark with distilled water.

#### *1 M Potassium monobasic phosphate (Solution B)*

Potassium monobasic phosphate ( $\text{KH}_2\text{PO}_4$ ) .....	13.61 gm
Distilled water....q.s. to.....	100.00 ml

Place the phosphate salt in a 100 ml volumetric flask, dilute with a portion of distilled water and dilute to the mark with distilled water.

#### *M/15 buffer solution*

Potassium dibasic phosphate solution, 1 M (Solution A) .....	43.0 ml
Potassium monobasic phosphate, 1 M (Solution B) .....	7.0 ml
Distilled water.....	700.0 ml

### **Buffered Water (buffered at various pH's) (16)**

#### *STOCK SOLUTIONS*

*Note:* These solutions keep indefinitely in refrigerator in pyrex glass stoppered bottle.

#### *M/15 Sodium dibasic phosphate (Solution A)*

Sodium dibasic phosphate ( $\text{Na}_2\text{HPO}_4$ ) .....	9.5 gm
Distilled water....q.s. to.....	1000.0 ml

Place the phosphate salt in a 1000 ml volumetric flask, dilute with a portion of distilled water and dilute to the mark with distilled water.

#### *M/15 Sodium monobasic phosphate (Solution B)*

Sodium monobasic phosphate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ) .....	9.2 gm
Distilled water....q.s. to.....	1000.0 ml

Place the phosphate salt in a 100 ml volumetric flask, dilute with a portion of distilled water and dilute to the mark with distilled water.

### WORKING SOLUTIONS

#### *Buffered water, pH 7.0*

Sodium dibasic phosphate, M/15 (Solution A).....	61.1 ml
Sodium monobasic phosphate, M/15 (Solution B).....	38.9 ml
Distilled water.....	900.0 ml

#### *Buffered water, pH 7.2*

Sodium dibasic phosphate, M/15 (Solution A).....	72.0 ml
Sodium monobasic phosphate, M/15 (Solution B).....	28.0 ml
Distilled water.....	900.0 ml

#### *Buffered water, pH 6.4*

Sodium dibasic phosphate, M/15 (Solution A).....	27.0 ml
Sodium monobasic phosphate, M/15 (Solution B).....	73.0 ml
Distilled water.....	900.0 ml

### Bullard's Hematoxylin (17)

Alcohol, ethyl, 50 percent.....	144.0 ml
Glacial acetic acid ( $\text{CH}_3\text{COOH}$ ).....	34.0 ml
Hematoxylin crystals.....	8.0 gm
Distilled water.....	250.0 ml
Ammonium alum ( $\text{AlK}(\text{SO}_4)_2 \cdot 2\text{H}_2\text{O}$ ).....	60.0 gm
Mercuric oxide, red ( $\text{HgO}$ ).....	8.0 gm
Alcohol, ethyl ( $\text{C}_2\text{H}_5\text{OH}$ ), 95 percent....	275.0 ml
Glycerin ( $\text{C}_3\text{H}_5(\text{OH})_3$ ).....	330.0 ml

Mix the 50 percent alcohol, 16 ml of the glacial acetic acid, and hematoxylin crystals, and bring to near boiling. Add the 250 ml of distilled water and 20 gm of the ammonium alum and bring the solution to a boil. Add the red mercuric oxide, cool quickly and filter. Add the 95 percent alcohol and the glycerin together with the remaining 18 ml of glacial acetic acid and 40 gm of ammonium alum. Ripen in bright sunlight for about a week and filter again before use.

### Carbol-Xylo (18)

Xylo.....	300 ml
Phenol ( $\text{C}_6\text{H}_5\text{OH}$ ), melted crystals.....	100 ml

### Carnoy's Fluid (19)

Alcohol, ethyl, absolute ( $\text{C}_2\text{H}_5\text{OH}$ ).....	60 ml
Chloroform ( $\text{CHCl}_3$ ).....	30 ml
Glacial acetic acid ( $\text{CH}_3\text{COOH}$ ).....	10 ml

### Culture Medium for Amoebae Occurring in Coldblooded Animals (20)

Gastric mucin.....	75.00 mg
Sodium chloride ( $\text{NaCl}$ ).....	1.50 gm
Sodium dibasic phosphate, M/15.....	21.75 ml
Potassium monobasic phosphate, M/15.....	3.25 ml
Distilled water.....	225.00 ml

Place the distilled water and the two salt solutions in a 500 ml beaker. Add the gastric mucin and heat with constant stirring for about 5 minutes to dissolve mucin. Tube the medium in 5 ml amounts in 15 ml test tubes. Plug the test tubes with absorbent cotton and sterilize the medium at 15 pounds pressure for 20 minutes. Following sterilization, cover cotton plugs with melted paraffin or a mixture of equal parts of vaseline and paraffin to seal the tubes and prevent dehydration. Store medium in the refrigerator.

Just prior to inoculating the medium, 2 or 3 loopfuls of sterile rice starch should be added to each tube. To inoculate fresh tubes, transfer 2 or 3 drops of the mucoid sediment from old cultures. Incubate at room temperature. Subcultures should be made at approximately 10-day intervals. Active trophozoites are best observed at the end of the first week after the inoculation of the medium.

### Delafield's Hematoxylin (21)

Hematoxylin crystals.....	1 gm
Alcohol, ethyl, absolute ( $\text{C}_2\text{H}_5\text{OH}$ ).....	10 ml
Saturated solution of aluminum ammonium sulphate ( $\text{NH}_4\text{Al}(\text{SO}_4)_2$ ) in distilled water.....	100 ml
Glycerin ( $\text{C}_3\text{H}_5(\text{OH})_3$ ).....	25 ml
Alcohol, methyl, absolute ( $\text{CH}_3\text{O}$ ).....	25 ml

Dissolve the hematoxylin crystals in the absolute ethyl alcohol and add a few drops at a time to the saturated ammonium alum solution. Leave this solution unstoppered in direct sunlight or in a 37°C. incubator for several weeks to oxidize the hematoxylin to hematin. When ripened, filter and add the glycerin and methyl alcohol, and the stain is then ready for use.

### Dichromate-Acid Clean Solution (for General Use) (22)

Sulfuric acid ( $\text{H}_2\text{SO}_4$ ), concentrated, commercial.....	1,000 ml
Sodium dichromate ( $\text{Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$ ), technical grade saturated aqueous solution.....	35 ml

Always pour the acid into the dichromate solution. In mixing these two solutions, excessive heat and spattering of the solution will occur if this precaution is not taken. Vessels in which the cleaning solution is placed should be submerged in a sand box of sufficient size to allow a margin of approximately eight inches from the edge of the vessel to the edge of the sand box. The layer of sand should be four to six inches in depth.

#### Dichromate-Acid Cleaning Solution (for Slides) (23)

Potassium dichromate ( $K_2Cr_2O_7$ ) . . . . .	50 gm
Commercial sulfuric acid ( $H_2SO_4$ ) . . . . .	50 ml
Tap water . . . . .	500 ml

This solution is especially recommended for cleaning used slides. Slides are immersed in this solution for 24 hours and the fluid is then poured off. Slides are rinsed thoroughly in tap water to remove all traces of the cleaning solution. Slides are then washed in a mild soap solution, rinsed in tap water, followed by a rinse in distilled water, and covered with 95 percent ethyl alcohol. The slides are then dried with a lintless starch-free cloth.

#### Ehrlich's Acid Hematoxylin (24)

Hematoxylin crystals . . . . .	2 gm
Glacial acetic acid ( $CH_3COOH$ ) . . . . .	10 ml
Alcohol, methyl, absolute ( $CH_3O$ ) . . . . .	100 ml
Glycerin ( $C_3H_5(OH)_3$ ) . . . . .	100 ml
Distilled water . . . . .	100 ml
Aluminum potassium sulphate ( $AIK(SO_4)_2 \cdot 12H_2O$ ) . . . . .	10 gm

Add the glacial acetic acid to 25 ml of the absolute alcohol. Dissolve the hematoxylin in the latter solution and add the remaining 75 ml of methyl alcohol and the glycerin. Heat the distilled water and dissolve the potassium alum in this solution. While still warm, slowly add the hematoxylin solution to the solution of potassium alum. Leave this solution unstoppered in direct sunlight or in a 37° C. incubator for several weeks to oxidize the hematoxylin to hematin.

#### Field's Stain Solution A (25)

Methylene blue . . . . .	0.80 gm
Azure B (American stains) . . . . .	0.50 gm
Anhydrous disodium phosphate ( $Na_2HPO_4$ ) . . . . .	5.00 gm

Monobasic anhydrous potassium phosphate ( $KH_2PO_4$ ) . . . . .	6.25 gm
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Distilled water . . . . .	500.00 ml
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Dissolve phosphate salts and add the powdered stains. Solution of Azure B is better accomplished by grinding in a mortar with a small amount of the phosphate solution. Set the solution aside for 24 hours, filter, and it is ready for use.

#### Field's Stain Solution B (26)

Eosin . . . . .	1.00 gm
Anhydrous disodium phosphate ( $Na_2HPO_4$ ) . . . . .	5.00 gm

Monobasic anhydrous potassium phosphate ( $KH_2PO_4$ ) . . . . .	6.25 gm
Distilled water . . . . .	500.00 ml

Dissolve phosphate salts and add the powdered stain. Set the solution aside for 24 hours, filter, and it is ready for use.

#### Flemming's Fixative (27)

Osmic acid ( $H_2OsO_4$ ) . . . . .	0.2 gm
Chromic acid ( $H_2CrO_4$ ) . . . . .	1.5 gm
Distilled water . . . . .	190.0 ml
Glacial acetic acid ( $CH_3COOH$ ) . . . . .	10.0 ml

Add the glacial acetic acid to the distilled water and then add the osmic and chromic salts. Stir the solution at room temperature until thoroughly dissolved. This fixative should be prepared immediately before use. Caution: This fixative should be worked with under an exhaust hood since osmic acid fumes are highly toxic.

#### Gastric Juice, Artificial (28)

Hydrochloric acid (HCl), concentrated (35-37 percent) . . . . .	7-10 ml
Distilled water . . . . .	987 ml
Pepsin . . . . .	6 gm

Concentrations of pepsin and acid may vary considerably and the formula herein given is that which is suitable for the digestion of muscle tissue containing *Trichinella* larvae.

#### Giemsa's Stain (29)

Giemsa powder, CP . . . . .	0.6 gm
Glycerin, CP ( $C_3H_5(OH)_3$ ) . . . . .	50.0 ml
Alcohol, methyl, absolute, acetone-free ( $CH_3O$ ) . . . . .	50.0 ml

1. All glassware must be chemically clean and dry.
2. Measure 50 ml glycerin.

3. Weigh 0.6 gm Giemsa's stain (dry powder).
4. Place a small amount of dry stain in a mortar. Add a small amount of glycerin. Grind thoroughly. Pour off into a sterile flask. Repeat until all the stain has been ground with glycerin. Rinse the mortar and pestle with the remaining glycerin and pour into flask.
5. Place glycerin-dye mixture in 55°-60° C. water bath for 6-8 hours. Shake periodically.
6. Measure 50 ml methyl alcohol. Rinse mortar and pestle with a portion of this. Add all of the methyl alcohol (including that used to rinse mortar and pestle) to glycerin-dye mixture after the latter has been removed from water bath and allowed to cool to room temperature.
7. Stopper. Allow to age 2 weeks. After aging, filter into chemically clean, dry bottles.

### Gilson's Fixative (30)

Nitric acid, 80 percent.....	15 ml
Glacial acetic acid ( $\text{CH}_3\text{COOH}$ ).....	4 ml
Mercuric chloride ( $\text{HgCl}_2$ ).....	20 gm
Alcohol, ethyl, 60 percent.....	100 ml
Distilled water.....	800 ml

### Glucose-Glycerin for Mounting Nematodes (31)

Alcohol, methyl, absolute ( $\text{CH}_3\text{O}$ ).....	20 ml
Glucose ( $\text{C}_6\text{H}_{12}\text{OH}$ ).....	48 ml
Distilled water.....	52 ml
Glycerin ( $\text{C}_3\text{H}_5(\text{OH})_3$ ).....	10 ml

Dissolve the glucose in the distilled water and add glycerin and methyl alcohol. For preservation add a small lump of camphor.

### Glycerin-Alcohol (32)

Alcohol, ethyl, 70 percent.....	90 ml
Glycerin ( $\text{C}_3\text{H}_5(\text{OH})_3$ ).....	10 ml

### Glycerin Jelly (33)

Bacto-gelatin.....	15 gm
Glycerin ( $\text{C}_3\text{H}_5(\text{OH})_3$ ).....	50 ml
Chromium potassium sulfate ( $\text{CrK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ ), 1 percent solution.....	100 ml
Liquid phenol ( $\text{C}_6\text{H}_5\text{OH}$ ).....	1 ml
Distilled water.....	150 ml

Bring water to boil. Add gelatin and dissolve. Add glycerin and mix thoroughly. Add chromium potassium sulfate solution and liquid phenol. If glycerin jelly is to be used for taeniod ova, omit phenol. (Phenol blackens taeniod ova.) Dispense glycerin jelly in approximately 10 ml quantities in tubes. Stopper and keep in refrigerator.

### Heidenhain's Iron Hematoxylin (34)

Distilled water.....	100.0 ml
Hematoxylin crystals.....	0.5 gm
Alcohol, ethyl, 95 percent ( $\text{C}_2\text{H}_5\text{OH}$ )....	10.0 ml

Dissolve the hematoxylin crystals in the alcohol and then add to the distilled water. Age the stain as for Delafield's iron hematoxylin.

### Hemalum (35)

Hematoxylin.....	1.0 gm
Sodium iodate ( $\text{NaIO}_3$ ).....	0.2 gm
Alum ( $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ ).....	50.0 gm
Distilled water.....	1,000.0 ml

Dissolve the hematoxylin in the water. Add the sodium iodate and alum. Dissolve and filter. This solution does not keep very well. It may be made more stable by adding 50 gm of chloral hydrate and 1 gm of citric (or acetic) acid.

### Hematoxylin, 0.5 Percent Solution (36)

Hematoxylin crystals.....	0.5 gm
Distilled water.....	100.0 ml

### Iodine Alcohol (for hematoxylin stain for protozoa) (37)

Alcohol, ethyl, 70 percent.....	100 ml
Saturated solution of iodine in 70 percent alcohol.....	5 ml

The exact concentration of iodine is not important; however, iodine must be in excess and as long as the iodine color persists, the concentration of iodine is adequate.

### Iodine-Acetic Acid Solution (38)

Potassium iodide (KI).....	1.0 gm
Iodine crystals.....	0.5 gm
Distilled water.....	100.0 ml
Glacial acetic acid ( $\text{CH}_3\text{COOH}$ ).....	1.0 ml

### Iodine Solution (D'Antoni's) (39)

- A. Preparation of 10 percent standardized potassium iodide solution:

- Place 100 gm of potassium iodide in a 1,000 ml volumetric flask, add distilled water, and allow crystals to dissolve. Q. s. to 1,000 ml mark.
- Carefully weigh to fourth decimal place a clean, dry 25 ml volumetric flask. Record weight.
- Fill 25 ml flask to mark with solution prepared in (1).
- Weigh filled flask and subtract original weight obtained in (2). Theoretically, the 25 ml of solution should weigh 26.925 gm, however, due to deliquescence of potassium iodide, it will actually weigh somewhat less. For example, 26.525 gm.
- Determine the difference between theoretical and actual weight.

*Example:*  $\frac{26.925 \text{ gm theoretically}}{26.525 \text{ gm actually}}$   
 $\quad \quad \quad .400 \text{ difference in weight}$

- Express this difference in percent by dividing the difference in weight by the theoretical weight and multiply by 100.

*Example:*  $\frac{.400}{26.925} \times 100 = 1.485 \text{ percent difference.}$

- Determine actual percentage of original solution by subtracting the percentage difference in weight from the desired 10 percent.

*Example:* 10.000 percent desired  
 $\quad \quad \quad 1.485 \text{ percent difference}$   
 $\quad \quad \quad 8.515 \text{ percent actual percentage of original solution}$

- Determine the amount of potassium iodide that should have been added to have given the desired 10 percent solution by the following arithmetical proportion:

$$\frac{100 \text{ gm KI originally added}}{x \text{ (gm of KI that should have been added)}} = \frac{\text{actual percent solution}}{\text{desired 10 percent solution}}$$

OR

$$\frac{100 \text{ gm}}{x \text{ gm}} = \frac{8.515 \text{ percent}}{10.000 \text{ percent}}, \text{ solving, } x = 117 \text{ gm}$$

- Subtracting the 100 gm originally added from the amount obtained in (8) above gives the amount that should be added to the original 1,000 ml to give the desired 10 percent standardized solution of potassium iodide.

*Example:* 117 gm  
 $\quad \quad \quad - 100 \text{ gm}$   
 $\quad \quad \quad \underline{17 \text{ gm KI to be added}}$

- Return the 25 ml of solution to the original flask. Add the amount of KI obtained in (9) to the 1,000 ml of solution prepared originally and allow it to dissolve. This standardized solution remains stable as long as it is tightly stoppered.

#### B. Preparation of the iodine stain

- Transfer of 10 ml of KI standardized solution into a 100 ml volumetric flask and fill to mark with distilled water.
- Add 1.5 gm of powdered iodine crystals to the 100 ml of KI solution.
- Let stand about 4 days with occasional agitation and filter the solution into dark colored bottle. Keep stoppered tightly when not in use. This solution will remain satisfactory for several weeks if tightly stoppered and protected from light. Discard and make new stain when it fails to stain sharply.

#### Iodine Solution (Dobell and O'Connor's) (40)

Iodine (powdered crystals).....	1 gm
Potassium iodide (KI).....	2 gm
Distilled water.....	100 ml

Mix ingredients and filter. This solution should be freshly prepared about every 10 days.

#### Iodine-Eosin Solution (Donaldson's) (41)

Physiological saline (0.85 percent).....	2 ml
Saturated solution of eosin in physiological saline.	2 ml

Iodine solution <sup>1</sup> .....	1 ml
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<sup>1</sup> Formula: Physiological saline 100 ml, potassium iodide 5 gm, powdered iodine crystals to saturation.

#### Iodine Solution for Staining Protozoa (U.S. Army Method) (42)

Iodine crystals.....	2 gm
Potassium iodide (KI).....	4 gm
Distilled water.....	100 ml

This solution should be freshly prepared about every 10 days. As the stain gets older, there is a subtle, not easily recognized loss in differentiation in the stained cysts.

#### Iodine Solution (Lugol's) (43)

Iodine (powdered crystals).....	5 gm
Potassium iodide (KI).....	10 gm
Distilled water.....	100 ml

Mix ingredients and filter. This stock solution will remain satisfactory for months. Before use, dilute with 5 parts of distilled water. Prepare fresh solution from stock about every 10 days.

#### JSB Stain Solution A(44)

Medicinal methylene blue.....	0.5 gm
Potassium dichromate ( $K_2Cr_2O_7$ ).....	0.5 gm
Sulfuric acid, 1 percent (1 ml conc. $H_2SO_4$ plus 99 ml water).	2.5 gm
Water.....	500.0 ml
Potassium hydroxide (KOH), or sodium hydroxide (NaOH), 1 percent.	10.0 ml

Dissolve the methylene blue thoroughly in 500 ml water. Add the 1 percent sulfuric acid, mix thoroughly, and add the potassium dichromate. A heavy amorphous purple precipitate of methylene blue chromate forms. Heat in an autoclave at a temperature of 100°–109° C. and at a pressure of 0–5 pounds for 3 hours. If the color remains greenish, further heating for another hour or so is required. At the end of this period, the solution turns blue which indicates almost complete polychroming. If the temperature is allowed to rise above 110° C., the oxidation of methylene blue may be carried too far and the solution will turn violet purple. When the solution has turned deep blue, allow it to cool to room temperature. Then add 10 ml of 1 percent potassium hydroxide or sodium hydroxide slowly, drop by drop, while constantly shaking the flask. After the total amount of the alkali has been added, transfer half the contents of the flask into another of the same capacity and continue to shake for 15 or more minutes. Pour from one flask to the other until the precipitate is dissolved and the solution has turned a deep blue with a violet iridescence. Leave at room temperature for 48 hours to mature, and filter.

#### JS B Stain Solution B(45)

Dissolve 1 gm of water soluble eosin in 500 ml tap water. Set aside 48 hours and filter.

#### Lacto-Phenol (46)

Glycerin ( $C_3H_5(OH)_3$ ).....	20 ml
Distilled water.....	10 ml
Phenol, melted crystals ( $C_6H_5OH$ ).....	10 ml
Lactic acid ( $C_3H_6O_3$ ).....	10 gm

#### Locke's Solution (47)

Sodium chloride ( $NaCl$ ).....	9.00 gm
Sodium bicarbonate ( $NaHCO_3$ ).....	0.20 gm
Potassium chloride ( $KCl$ ).....	0.42 gm
Calcium chloride ( $CaCl_2$ ).....	0.25 gm
Dextrose ( $C_6H_{12}O_6$ ).....	2.00 gm
Distilled water.....	1000.00 ml

Calcium chloride should be dissolved in a little of the water and added to the remainder of the solution to prevent precipitation.

#### Mann's Stain for Protozoa (48)

Methylene blue.....	0.35 gm
Eosin.....	0.45 gm
Distilled water.....	180.00 ml

#### Mayer's Hydrochloric Acid-Carmine (49)

Carmine.....	4 gm
Water.....	15 ml
Hydrochloric acid ( $HCl$ ), concentrated.....	20 drops
Alcohol, ethyl ( $C_2H_5OH$ ), 95 percent.....	95 ml

Mix the dye, water, and acid and boil until the dye is completely dissolved. Add the 95 percent alcohol, filter, and neutralize with ammonia almost to precipitation point and refilter. For use, the stock stain is diluted 1 in 10 with 70 percent alcohol to which a few drops of hydrochloric acid have been added.

#### Mayer's Paracarmine (50)

Carminic acid ( $C_{12}H_{11}O_7$ ).....	1.0 gm
Aluminum chloride ( $AlCl_3 \cdot 6H_2O$ ).....	0.5 gm
Calcium chloride ( $CaCl_2$ ).....	4.0 gm
Alcohol, ethyl, 70 percent.....	100.0 ml

#### Merthiolate-Iodine-Formaldehyde Stain-Preservative (51)

The following proportions of ingredients compensate for the gradual deterioration of the Lugol's solution. (Time in weeks refers to age

of Lugol's solution. The latter should be freshly prepared every 3 weeks.)

Week	Lugol's solution	Formaldehyde (USP)	Tincture merthiolate (Lilly 1:1000)
First.....	10.0	12.5	77.5
Second.....	12.5	12.5	75.0
Third.....	15.0	12.5	72.5

### Methyl Green-Pyronin (52)

Methyl green.....	2 gm
Pyronin.....	1 gm
Distilled water.....	300 ml

### Mordant (Hematoxylin Stain for Protozoa) (53)

Ferric ammonium sulfate ( $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ ) (violet crystals).....	4.00 gm
Glacial acetic acid ( $\text{CH}_3\text{COOH}$ ).....	1.00 ml
Sulphuric acid ( $\text{H}_2\text{SO}_4$ ), concentrated (sp. gr. 1.84).....	0.12 ml
Distilled water.....	100.00 ml

### Normal Saline (Physiological Saline) (54)

Sodium chloride CP ( $\text{NaCl}$ ).....	0.85 gm
Distilled water.....	100.00 ml

For parasites of cold-blooded vertebrates use 0.7 gm of sodium chloride to each 100 ml of distilled water.

### Phosphotungstic Acid-Hematoxylin Stain (55)

Hematoxylin.....	0.10 gm
Phosphotungstic acid ( $\text{H}_3\text{PO}_4 \cdot 12\text{WO}_3$ ).....	2.00 gm
Distilled water.....	100.00 ml
Potassium permanganate (optional, for rapid ripening of stain) ( $\text{KMnO}_4$ ).....	0.25 gm

Dissolve powdered hematoxylin in 20 ml of distilled water with gentle heating. Allow the solution to cool, then add distilled water to a total volume of 80 ml. Dissolve 2 gm phosphotungstic acid in 20 ml of distilled water at room temperature and mix this solution with the hematoxylin solution.

Allow the stain to age for several months before use. For immediate use the stain may be artificially ripened by adding 10 ml of a 0.25 percent aqueous potassium permanganate solution. To prepare this solution dissolve 0.25 gm

of potassium permanganate crystals in 100 ml of distilled water. Care must be taken in preparing this solution, as an excess concentration will cause precipitation of the total volume of prepared stain, rendering it useless for staining purposes.

### PVA-Fixative Preparation (56)

Powdered PVA (Elvanol 90-25) .....	25.0 gm
Mercuric chloride solution, saturated ( $\text{HgCl}_2$ ) .....	312.0 ml
Glycerin ( $\text{C}_3\text{H}_5(\text{OH})_3$ ) .....	7.5 ml
Glacial acetic acid ( $\text{CH}_3\text{COOH}$ ) .....	25.0 ml
Alcohol, ethyl, 95 percent ( $\text{C}_2\text{H}_5\text{OH}$ ) .....	156.0 ml

For mixing, a high speed agitator which will produce a vortex or whirlpool in the fixative solution is highly desirable. However, manual stirring of a character to produce the aforementioned whirlpool is satisfactory. The mixing container and stirring rod should be corrosive-resistant, as should all other objects coming in contact with the PVA-fixative.

Mix the mercuric chloride solution, glycerin, glacial acetic acid, and ethyl alcohol thoroughly. At room temperature slowly sift in the PVA with constant stirring or whirling in an agitator. Stir or agitate for 10 minutes and then place the mixture in a water bath heated to  $75^\circ$ - $85^\circ$  C. Stir while heating for an additional 5-10 minutes or until solution is complete.

*Note:* Elvanol 90-25 may be obtained from Delkote, Inc., P.O. Box 1335, Wilmington, Delaware, in small quantities. Suitable substitutes Elvanol, grades 71-24 and 71-20 can be obtained from E. I. Dupont de Nemours and Co., Electrochemical Dept., Niagara Falls, New York. This does not constitute an indorsement of these products and there are likely others which have not been tested which could serve as acceptable substitutes.

### Quensel's Solution (57)

Sudan III, saturated solution in 80 percent ethyl alcohol.....	20 ml
Methylene blue, medicinal (saturated aqueous filtered solution) .....	30 ml
Cadmium chloride, CP, 10 percent aqueous solution.....	50 ml
Distilled water.....	250 ml

Mix the Sudan III and methylene blue solutions, shake and filter into the solution of cadmium chloride. Occasionally shake this mixture over a period of 15-20 minutes until

precipitation is completed. Filter and leave the precipitate on the filter paper for 24 hours. Transfer precipitate to a fresh filter paper and wash through with 25–30 ml of distilled water. Transfer precipitate to the 250 ml of distilled water. If fine crystals should precipitate after a few days, filter this solution once more.

### **Reynold's Mixture (58)**

Alum cochineal.....	30 ml
Delafield's hematoxylin (aged).....	10 ml
Distilled water.....	250 ml

Mix and allow to stand several hours. A fine precipitate will settle out which should be removed by filtering. The mixture is then ready for use.

### **Ringer's Solution (59)**

Sodium chloride (NaCl).....	8.0 gm
Potassium chloride (KCl).....	0.2 gm
Calcium chloride (CaCl <sub>2</sub> ).....	0.2 gm
Magnesium chloride (MgCl <sub>2</sub> ).....	0.1 gm
Monosodium phosphate (NaH <sub>2</sub> PO <sub>4</sub> ).....	0.1 gm
Sodium bicarbonate (NaHCO <sub>3</sub> ).....	0.4 gm
Distilled water.....	1000.0 ml

To prepare sterile Ringer's solution dissolve the first four ingredients in 900 ml of the distilled water and autoclave at 15 pounds pressure for 20 minutes. Dissolve the sodium phosphate and sodium bicarbonate in 100 ml of sterile distilled water. Seitz filter, and add to the autoclaved solution.

### **Ristroph's Fixative for Cestodes (60)**

Formalin, commercial (HCHO).....	100 ml
Alcohol, ethyl (C <sub>2</sub> H <sub>5</sub> OH), 95 percent.....	250 ml
Glacial acetic acid (CH <sub>3</sub> COOH).....	50 ml
Glycerol (C <sub>3</sub> H <sub>8</sub> (OH) <sub>3</sub> ).....	100 ml
Distilled water.....	500 ml

### **Saline-Iron-Hematoxylin Solution (NAVMED) (61)**

Physiological saline (0.85 percent).....	75.00 ml
Hematoxylin, 0.5 percent.....	15.00 ml
Ferric ammonium sulphate, 4 percent.....	0.25 ml

This solution coagulates fecal particles, thus freezing the organisms, but still permits normal activity of the trophozoites and increases their refractiveness by contrast with the stained background.

### **Saponin Solution (62)**

Saponin.....	0.2 gm
Physiological saline.....	100.0 ml

This solution is used to hemolyze blood. To hemolyze blood, add 10 ml of this solution to each ml of blood.

### **Schaudinn's Fixative (63)**

Mercuric chloride, saturated solution in distilled water (HgCl <sub>2</sub> ).....	200 ml
Alcohol, ethyl, 95 percent (C <sub>2</sub> H <sub>5</sub> OH).....	100 ml
Glacial acetic acid (CH <sub>3</sub> COOH).....	15 ml

Glacial acetic acid should be added just prior to use at the rate of 5 ml for each 95 ml quantity of the Schaudinn's stock solution. The acidified Schaudinn's deteriorates and should be prepared fresh each time it is used.

### **Semichon's Carmine (Stock Solution) (64)**

Glacial acetic acid (CH <sub>3</sub> COOH).....	100 ml
Distilled water.....	100 ml
Carmine.....	q.s.

Mix the acetic acid with the distilled water and add carmine in excess. Heat this solution to 95°–100° C. for 15 minutes, cool, and filter. Dilute stock solution with an equal amount or more of 70 percent alcohol before use.

### **Sodium Hydroxide Solution for Stoll Count (65)**

Sodium hydroxide (NaOH).....	4 gm
Distilled water.....	1000 ml

### **Sodium Hyposulfite Solution for Removing Iodine (66)**

Sodium hyposulfite (Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> ·5H <sub>2</sub> O).....	5 gm
Distilled water.....	100 ml

Dissolve the sodium hyposulfite in the distilled water. Each time a new series of staining jars are prepared add 2 or 3 ml of this solution to each 50 ml or 70 percent alcohol used in the first dish after iodized alcohol in the phosphotungstic acid hematoxylin technique.

### **Tannic Acid Fixative (Alli) (67)**

Tannic acid, CP(C <sub>7</sub> H <sub>6</sub> O <sub>7</sub> ).....	4 gm
Alcohol, ethyl, 95 percent (C <sub>2</sub> H <sub>5</sub> OH).....	90 ml
Glacial acetic acid (CH <sub>3</sub> COOH).....	5 ml
Phenol, liquefied (C <sub>6</sub> H <sub>5</sub> OH) .....	1 ml

### **Tergitol-Hematoxylin (Stock Solution) (68)**

Hematoxylin crystals.....	0.5 gm
Alcohol, ethyl, 95 percent ( $C_2H_5OH$ ).....	10.0 ml
Tergitol, #7.....	q.s. ml

To prepare the stock hematoxylin solution, dissolve the hematoxylin crystals in the 95 percent alcohol. To prepare the stain, add 90 ml of distilled water. Age the stain for 1 month in an unstoppered bottle exposed to sunlight or ripen artificially by adding a minute quantity of sodium bicarbonate. To every 30-40 ml of this solution add 1 drop of tergitol #7 just before use. Tergitol-hematoxylin should be prepared fresh every 24 hours.

### **Trichrome Stain (69)**

Chromotrope 2R.....	0.60 gm
Light green SF.....	0.15 gm
Fast green FCF.....	0.15 gm
Phosphotungstic acid ( $H_3PO_4 \cdot 12WO_3$ ).....	0.70 gm
Glacial acetic acid ( $CH_3COOH$ ).....	1.00 ml
Distilled water.....	100.00 ml

Place distilled water in 250 ml beaker and add the glacial acetic acid. At room temperature add remaining reagents in order listed stirring after each reagent is added until it is thoroughly dissolved. Stored in a tightly stoppered bottle this stain will keep indefinitely.

### **Velat-Weinstein-Otto Solution (70)**

1. Make up a 2.5 percent solution of crystal violet in warm distilled water.
2. Make up a 1 percent solution of hematoxylin in boiling distilled water. Add 0.4 ml. of triethanolamine to 100 ml of the boiling hematoxylin solution; boil for 2-3 minutes, then cool to approximately 50° C.
3. Add entire volume of warm hematoxylin-triethanolamine solution to solution No. 1, with constant stirring. This will result in rapid formation of a precipitate.
4. Filter several times through Whatman No. 2 filter paper. Save the precipitate on the filter paper and discard the filtrate. Wash down the precipitate into the cone of the filter paper, using small amounts of distilled water, totaling not more than 50 ml. Allow the precipitate to dry.

5. The dried precipitate is made up as a 0.05 percent solution in acetate buffer which is obtained by combining (a) 16.82 ml glacial acetic acid in 983.18 ml distilled water with (b) 19.72 gm sodium acetate ( $NaC_2H_3O_2 \cdot H_2O$ ) in 980.28 ml distilled water, as follows: for pH 4.6 use 102 ml solution (a) and 98 ml solution (b); for pH 4.8, 80 ml and 120 ml; for pH 5.2, 42 ml and 158 ml, and for pH 5.4, 29 ml and 171 ml. Two to three weeks, with frequent shaking, are required to dissolve the precipitate. The solution is then filtered and the undissolved precipitate is discarded.

### **Willis-Malloy Brine Solution (71)**

Prepare the brine solution by stirring sodium chloride into hot or boiling tap water until the excess added will not go into solution. This saturated solution should be checked with a hydrometer to insure that the specific gravity is at least 1.20. It is best to filter before using.

### **Wright's Stain (72)**

Wright's stain powder.....	0.3 gm
Glycerol neutral, CP( $C_3H_5(OH)_3$ ).....	3.0 ml
Alcohol, methyl, absolute, acetone-free ( $CH_3O$ ).....	97.0 ml

Grind powder in a mortar. Add glycerol and grind thoroughly. Add the methyl alcohol and mix. Let stand overnight in tightly stoppered container. Filter. Allow to age for a few days before use. The stain improves with age. Store in dark colored bottle.

### **Xylol-Damar (73)**

The ratio of xylol and damar will be determined by the consistency of mounting medium desired. Select clean white lumps of damar resin and add a sufficient quantity of xylol to make a solution about the consistency of maple syrup. Let this stand for several hours and then filter through a paper towel moistened with xylol. Place the filtrate in a beaker, cover securely with a gauze tied over the mouth of the beaker, and set aside in a 37° C. incubator until a sufficient amount of the xylol has evaporated off to give medium of the desired consistency.

**Zenker's Fixative (74)**

Sodium sulphate ( $\text{Na}_2\text{SO}_4$ ) . . . . .	1.0 gm
Mercuric chloride ( $\text{HgCl}_2$ ) . . . . .	5.0 gm
Potassium dichromate ( $\text{K}_2\text{Cr}_2\text{O}_7$ ) . . . . .	2.5 gm
Distilled water . . . . .	100.0 ml

Glacial acetic acid is added at the rate of 5 ml for each 95 ml of the above solution just before use. Use the solution at once after addition of the acetic acid. Discard after each use and prepare freshly acidified fixative each time material is run up.

**Zinc Sulfate Solution for Concentration of Protozoan Cysts (75)**

Zinc sulfate, USP ( $\text{ZnSO}_4$ ) . . . . .	331 gm
Tap water (warm) . . . . .	1000 ml

Dissolve thoroughly, check the specific gravity with a hydrometer. It should read 1.18. Adjust, if necessary, by adding water or zinc sulfate as needed. To make a solution of specific gravity 1.20, add zinc sulfate until this reading is obtained.



## Appendix 2

# MAINTENANCE OF PARASITE CYCLES IN LABORATORY ANIMALS AND CULTURE

Several medically important parasites are readily maintained in the laboratory for the purpose of demonstrating various stages in their life cycles. Organisms so maintained serve as an excellent source of material for permanent mounts and also afford the opportunity for study of parasites in the living state. Additionally, they provide valuable material for training purposes.

The maintenance of cycles in the laboratory is not without pitfalls and the more important of these will be emphasized here. Successful maintenance of the cycles requires proper attention in order to prevent their loss through oversight or neglect. A paramount rule is never to blindly transfer material which is presumed to be infective without examination to verify that the parasites or the desired stages in their cycles are present in material to be inoculated. Many infections are self-limiting and after a period of time, the parasites are eliminated and the host animal may be immune or quite refractive to reinfection. Some of the parasites covered in this chapter occur

as natural infections of wild or domesticated laboratory animals. When dealing with parasites which fall in this category, only animals reared in the laboratory and adequately protected against natural infections should be utilized. In some cycles, the time interval between successive transfers is critically important. For self-limiting infections as well as those which ultimately prove fatal, a definite time table should be set up and rigidly followed. As an added assurance against loss of cycles, those which can be cultured should be simultaneously maintained in culture and in host animals. In handling material pathogenic for man, adequate precautions must be taken to avoid human infection. Carcasses of animals harboring pathogens should be destroyed by incineration. Infected animals should be housed in separate animal quarters and accurately identified with complete records. It is a convenient expedient to identify infected animals with different colored dyes using a distinctive color for marking animals harboring each of the cycles maintained in the laboratory.

## MAINTENANCE OF CERTAIN PROTOZOAN CYCLES

### **Trypanosoma Cruzi**

*T. cruzi*, the causative organism of Chagas' disease can be maintained in various laboratory animals including mice, rats, and guinea pigs. The discussion presented here will be limited to the technique for maintaining *T. cruzi* in Carworth Farms White strain albino mice. Before attempting passage, first ascertain that trypanosomes are present in the circulating blood. The infection is passed from infected to susceptible animals by intraperitoneal

inoculation of several drops of blood in about 0.25 ml of normal saline per mouse. It should be passed either simultaneously with each initial demonstration of parasites in the blood of recently infected animals or routinely every 21 days from verified positives. Inoculate two or three animals each time material is passed.

With strains of *T. cruzi* which no longer produce fatal infections, the organisms can generally be recovered from newly infected animals between 14 and 21 days following in-

oculation. To obtain a blood specimen for examination, cut the tip of the tail of the mouse and force a drop of blood out onto a slide on which a drop of normal saline has been placed. With an applicator stick thoroughly mix the blood and saline, coverslip, and examine under high dry magnification adjusting illumination to facilitate observation of the hyaline motile forms. Oftentimes the parasites are present in such limited numbers that very careful search is required to detect them. In what could be considered comparatively heavy infections parasites rarely exceed three or four per high power field.

A number of different strains of *T. cruzi* are now maintained in various laboratories in the United States. These vary in pathogenicity, duration of parasitemia, and parasite density in the blood of infected animals. Strain characteristics are subject to change and it is only by experience that one can learn how a strain may be expected to behave. The strain of *T. cruzi*, the age of animals employed, and the strain of experimental mice utilized all affect the course of the infection. Experience with the Chile strain of *T. cruzi* is cited as an example. Initially, this strain killed mice 6 weeks of age in about 10 days following inoculation. When this strain began to show signs of loss of virulence as evidenced by longer survival of the mice, mice 3 weeks of age were inoculated and virulence was again stepped up. After a few passages in the younger mice, the strain again commenced to display loss of virulence with survival and recovery of infected mice. Mouse adaption eventually reached a point where, regardless of age of mice used, the animals invariably survived the infection and were refractive to reinfection.

If *T. cruzi* is maintained in culture, it tends to lose infectivity for animals with each successive transfer and after 3-4 transfers, it is generally no longer capable of producing demonstrable parasitemia. Where cultures are maintained for the purpose of infecting animals with *T. cruzi* repassage into mice should be intermittently carried out after 3 or fewer serial passages in culture. Organisms in the leptomonad stage are usually quite plentiful in culture by the end of the second week fol-

lowing inoculation of fresh medium and the cultures can be safely retained 6 weeks between subcultures. Preparation of media and trypanosome culture are discussed elsewhere in this manual.

### Trypanosoma Lewisi

The common rat trypanosome, *T. cruzi*, is readily transmissible to laboratory rats. The infection does not appear to be injurious to these animals and is self-limiting in the rat with duration of parasitemia generally varying from 2-6 weeks. To insure against loss of the cycle, it should be transferred not less frequently than every 14 days. The infection can be passed either directly by inoculation of blood from an infected animal, by passage of culture from the fluid overlay, or through the bite of the rat flea *Ceratophyllus fasciatus*. It is easily maintained in culture utilizing techniques described elsewhere. If it is desired to maintain pathogenicity for rats, the number of successive transplants on culture media should not exceed 3 prior to repassage into the natural warm-blooded host.

To pass the infection from one animal to another, cut the tip of the tail of the infected animal and immerse it in a Wassermann tube containing 1 or 2 ml of physiological saline. Massage the tail to enhance blood flow and draw off sufficient blood to render the saline and blood suspension a bright pink color. After verifying the presence of the organism by microscopic examination, draw up the blood-saline suspension into a 2 ml syringe fitted with a 23-gauge,  $\frac{3}{4}$ -inch needle. Inoculate 0.5 ml quantities of the blood-saline suspension into each rat via the intraperitoneal route. To pass the infection to rats from culture media, inoculate 0.1 ml of fluid from the liquid overlay in the same manner. Following inoculation of culture fluid or blood containing trypanosomes highest parasite density is usually reached in the new host in from 5-7 days. To maintain organisms in culture, passage to new media should be accomplished at least every 6 weeks. Transplants generally can be demonstrated to contain considerable numbers of organisms in the leptomonad stage about 14 days after inoculation of the medium.

## **Leishmania Donovanii**

*L. donovani*, the causative organism of kala-azar is readily maintained in the laboratory in hamsters. The infection is always fatal to the animals and in order to maintain the cycle, the hamsters must be sacrificed and the infection repassed periodically. Length of time of survival following inoculation is dependent principally upon two factors, namely, the age of the animals and the number of Leishman-Donovan bodies passed to the new host. Survival time generally varies between 60 and 90 days and the infection should, therefore, be passed every 6-8 weeks.

To infect a hamster, sacrifice an infected animal and place several portions of the spleen and liver in a sterile mortar. Verify whether or not the animal is positive by removing a small portion of spleen tissue and making several impressions on a glass slide. This is accomplished by lightly touching the slide at several points with the piece of tissue placed on the end of a tongue depressor. Without allowing the impressions to dry, the preparation is stained with either Giemsa's or Wright's stain using the same technique as described elsewhere for malaria parasites. Dry the preparation thoroughly and examine under oil immersion for typical intracellular Leishman-Donovan bodies within enlarged macrophage cells.

If the infection is verified, add about 2 ml of normal saline to the mortar containing the spleen and liver tissue and thoroughly macerate the tissue in the fluid. Passage to a new host is accomplished by inoculating 0.5 ml of the tissue suspension intraperitoneally utilizing a  $\frac{3}{4}$ -inch, 23-gauge needle. Sterile technique is not necessary in the handling of the inoculum since the normal response of the animal to bacterial contaminants is sufficient to destroy them.

## **Plasmodium Canthemerium**

Various species of bird malaria are maintained in laboratories throughout the United States, one of the more common being *P. canthemerium*. This species is readily transmissible to canaries, sparrows, and pigeons. If

birds do not succumb to the infection during the acute stage, they become solidly immune and refractive to reinfection with the same parasite. Following recovery from acute malaria, the infection becomes chronic in the birds and may remain so for several years. Birds can be infected either by passage of blood from birds in the acute or latent stage or through the agency of natural vectors.

To pass the infection from a bird harboring an acute or latent infection, draw up 0.1 ml of a 2 percent saline citrate solution into a 1 ml syringe fitted with a 25 or 27-gauge,  $\frac{1}{4}$ -inch needle. With a lancet, puncture the wing vein of the bird and as the blood wells up over the wound, draw it up into the syringe to the 0.5 ml mark. After the blood has been drawn, cover the wound with gel-foam or gauze to prevent excessive bleeding. Replace the short needle on the syringe with a 25-gauge, 2-inch needle, the outer third of which is bent back almost at right angles to the needle shaft. To inoculate intravenously, hold the bird to be injected firmly in the hand with one leg outstretched and insert the needle into the tarsal vein at a point just below the knee joint and inject the inoculum.

The infection can also be passed via the intramuscular or intraperitoneal route. If either of the latter techniques is utilized, the parasites do not appear as soon in the circulating blood as when the infection is passed intravenously. Utilizing the intravenous route, the peak of parasitemia is reached by the fifth to the seventh day following inoculation. By the other routes mentioned, the parasites usually can be detected in the blood about 2 weeks after inoculation.

If one of the natural vectors is utilized to transmit *P. canthemerium*, the mosquitoes must be allowed to feed on a bird in the acute stage of infection. *Culex quinquefasciatus* is easily colonized in the laboratory and serves as a very efficient vector. Larvae collected in the field should be placed in a beaker in a secure screen cage. Larvae can be fed on pelleted rabbit food, pablum, or bran. Two or 3 days following emergence of the adult mosquitoes, introduce a chick or rabbit into the cage to allow females to obtain a blood meal. Beakers of

tap water should then be placed in the cage to provide a suitable environment for oviposition. Females generally oviposit 48–72 hours following a blood meal. Egg rafts are harvested daily and placed in small pans containing 1–2 inches of tap water. At temperatures of 70° F. or higher, the eggs will hatch in 2 or 3 days. Feed larvae as indicated above. At normal room temperature, the larvae will pupate in 10–12 days and as soon as pupation begins to occur, the pupae should be pipetted off daily and transferred to beakers or other suitable vessels. The vessels are placed in screened cages where emergence will occur within 1 or 2 days at a temperature of 70° F. or above. Food in the form of sugar-water soaked wads of cotton should be placed in Petri dishes in the bottom of cages containing emerged adults. Twenty-four hours prior to the time the mosquitoes are to be allowed to feed on an infected bird, the sugar water should be removed.

Mosquitoes can be fed on birds in various ways. Two methods will be described here. A simple convenient procedure is to immobilize the bird by tying the feet together and the wings together and placing it in the bottom of the cage with the starved mosquitoes. A second method is to attach a burette clamp to a ring stand. Tie the bird's feet together and place it in a square piece of cloth of suitable size in which a hole has been cut away over the area where the belly of the bird will rest. Pluck the feathers over the belly of the bird to expose an area of skin which the mosquitoes can readily penetrate. Place the bird in the sling with the de-feathered area over the hole which has been cut out in the holding sling. Secure the free ends of the sling to the burette clamp, thus giving an arrangement which immobilizes the bird and provides a convenient mechanism whereby it can be raised or lowered. The belly of the bird is then brought into contact with the screened top of the cage by adjusting the position of the clamp on the ring-stand. *Culex quinquefasciatus* feeds avidly only darkness so when attempting to allow this species to feed in the daytime, the birds should be exposed to the mosquitoes in a darkened room. Birds can be immobilized for several

hours by either method without suffering any ill effects.

After exposure to a bird, engorged mosquitoes should be removed from the feeding cage with a suction tube and transferred to a new cage. Ten to 14 days following feeding, the sporozoites can be found in the salivary glands. To infect a bird through the bite of the mosquito, immobilize the bird and proceed as outlined above for the initial feeding. Malaria parasites generally appear in the blood of the bird 7–10 days following the bite of an infected mosquito.

During the acute stage of infection, it is possible to demonstrate gametocytes in the process of exflagellation and also the combination of gametes and production of zygotes. In the acute stage, large numbers of schizonts are present in the circulation, and all stages of schizogony are readily demonstrable if blood is drawn at different times over a 24-hour period. The schizogenous cycle is of 24 hours duration with rupture of erythrocytes and merozoite release taking place in late afternoon or in the evening.

To observe the process of exflagellation, obtain a drop of blood from a bird harboring an infection of about 10 days' duration and mix it with an equal quantity of physiological saline on a slide. Coverslip and allow the preparation to stand for about 15 minutes at room temperature and examine under high dry magnification. An alternative method is to obtain blood, mix with saline on a slide as indicated above, and place the slide in a Petri dish the bottom of which is covered with a water-soaked piece of gauze. The Petri dish is covered and set aside for 20 minutes at room temperature and then examined for exflagellating microgametocytes. The process of zygote formation can also be observed in such preparations.

To obtain sporozoites, dissect out the salivary glands of mosquitoes which have been fed on infected birds and held for a minimum of 10 days following infection. To remove the salivary glands, the head of the mosquito is severed from the body and transferred to a drop of saline on a slide. With dissecting needles,

the head is teased open and the glands are macerated. If present, the actively swimming spindle-shaped sporozoites are readily observed under high dry magnification. Oocysts can be demonstrated by teasing open the abdomen of

the mosquito with dissecting needles and exposing the stomach. They will appear as prominent gray nodules on the exterior surface of the stomach wall under high dry magnification.

## MAINTENANCE OF CERTAIN HELMINTH CYCLES

### **Hymenolepis Diminuta**

The common grain beetle, *Tenebrio molitor* is a most efficient intermediate host for *H. diminuta*. This beetle will develop up to 500 cysticercoids when properly fed on washed ova. The only disadvantage in working with this species is that the adult is fairly short-lived and infections must, therefore, be very carefully timed to avoid loss of the host before the cysticercoids are fully developed. Colonies of this beetle are easily maintained in the laboratory. They can be reared in screw-cap, large-mouth glass jars and fed on sterilized bran to which has been added a small amount of brewer's yeast. To maintain adequate moisture content, small bits of apple or raisins should be added to the bran-yeast mixture about once each week. To insure utilization of insects of known age, the beetle colony should be screened once each week and pupae transferred to clean rearing chambers. Females lay only one brood of eggs during their lifetime and the larvae take several months to pupate. Pupation does not occur during the summer months and adults are, therefore, usually available only between October and May. The total life span of *T. molitor* rarely exceeds 30 days in the adult state, and under ordinary conditions cysticercoids require 14-18 days to complete their development in the insect. It is, therefore, necessary to infect beetles which are not over 1 week of age.

*Tribolium confusum*, the common flour beetle also serves as intermediate host for *H. diminuta*. It has a life span of 2-3 months in the adult stage and can be reared the year round in the laboratory. Because of the longevity of this species, it is not necessary to screen the colony for pupae and the insects can be selected at random for infection. These beetles are considerably smaller than *T. molitor* and they rarely produce over 50 cysticercoids. Colonies with an extremely high density of beetles can be maintained on a mixture of bran and brewer's yeast or wheat flour moistened about once weekly with raisins or bits of apple.

Animals infected with *H. diminuta* pass eggs in large numbers, and they are usually quite uniformly distributed throughout the feces. Consequently, either grain beetles or flour beetles can be infected by feeding directly for 5-10 days on the freshly passed feces. This is done by placing a number of fresh moist fecal pellets on a filter paper in a Petri dish without supplying any other food. As many as 100 flour beetles or 25 grain beetles can be placed in the Petri dish at one time. Following above designated period of exposure to the fecal pellets, the insects are returned to normal diet.

Much higher rates of infection can be obtained in the beetles by feeding ova which have been concentrated from the feces. The beetles show a marked distaste for zinc sulphate so eggs should be concentrated according to the Willis-Malloy brine flotation technique. Infected rodents are placed in a clean cage the bottom of which is lined with white paper and the animals are left in the cage for several hours or overnight. Fecal pellets are then gathered into a container, distributed to a depth of about 1 inch in the bottom of centrifuge tubes, and concentrated according to the above-mentioned technique. Following concentration by flotation, the fluid adhering to the undersurfaces of the superimposed coverslips is washed off and pooled in another centrifuge tube and the tube is filled with tap water. The tube is then centrifuged at 1500 rpm for 2 minutes and the supernatant fluid decanted. Tap water is again added and cen-

trifuging repeated. Four or five washings are required to remove all traces of salt from the ova.

Following washing, the ova are pipetted from the bottom of the centrifuge tube with a capillary pipette and placed on a piece of filter paper about an inch to an inch and one-half in diameter. The piece of filter paper should be the same size as the diameter of the container in which the ova will be fed to the beetles. The paper containing the ova is placed on another sheet of filter paper and moved about to successive dry spots to draw off excess water from the egg-covered paper. To infect *T. molitor* a volume of scraped apple equal to the volume of ova is placed on the filter paper. With an applicator stick, the eggs are comminuted thoroughly in the mash and the mixture is spread evenly over the piece of filter paper. The beetles will search over the paper primarily for the sweetened water so all surfaces including the edges should be coated with the mixture. The undersurface of the paper is blotted several times more to draw off all moisture possible and is then placed in an appropriate-sized container. The desired number of grain beetles are placed on the egg-apple coated paper and the container is covered to protect against evaporation, dust, and insects.

To infect *T. confusum* concentrate ova, pool, and wash 4 or 5 times to remove all traces of salt. Place the ova on filter paper and dry very thoroughly by blotting the undersurface. Do not use apple as the sticky matrix will entrap these small beetles and quickly smother them by covering their spiracles. The filter paper to which the concentrated ova are transferred should be about an inch in diameter or smaller. The paper should be placed in the bottom of a 1-2 inch Stendor dish. Twenty-five to 50 beetles can be infected in this preparation. The beetles should be starved for about 2 weeks before being fed the ova.

Utilizing either the grain or the flour beetle as the intermediate host, the insects are exposed to the ova for 1 week after which they are put back on normal diet. Cysticercoids can be recovered from the insects 14-18

days after initial exposure to the ova. In the case of the short-lived *T. molitor* the insects should be dissected for cysticercoids not later than the eighteenth day after initial exposure. *T. confusum* can be expected to live approximately 2 months after initial exposure to the ova.

To recover the cysticercoids, place the beetle in a few drops of physiological saline in a watch glass. With dissecting needles, carefully tear the chitinous exoskeleton into small pieces. Agitate each piece with a needle to dislodge adhering cysticercoids and discard the debris. To pass the infection to a new host, place the watch glass under a binocular dissecting scope and with a capillary pipette, remove the desired number of mature cysticercoids. About 5 can be passed into a mouse and 10 into a rat for optimum egg production. The infective forms are introduced into the primary host by inserting the tip of the capillary pipette well down into the throat of the animal where they are discharged and swallowed. Ova can be recovered from infected animals about 1 month after infection.

### **Hymenolepis Nana**

The flour beetle, *T. confusum*, is the most efficient intermediate host for laboratory propagation of the dwarf tapeworm. This beetle will develop up to 100 cysticercoids when properly fed on ova concentrated from rodent droppings. The larger beetle, *T. molitor*, also serves as a suitable intermediate host but offers no advantages over the flour beetle since cysticercoid levels are about the same with this species as with *T. confusum*. For methods of colonizing these two species of insects refer to the preceding section on *H. diminuta*.

The life cycle of *H. nana* is unusual in that it may be successfully completed either with or without an intermediate host. Direct transfer of the infection to a new host may be accomplished either by force feeding macerated gravid proglottids, force feeding concentrated ova, or by exposing uninfected animals to droppings of infected animals in the uncleaned cages of rodents harboring the dwarf tapeworm.

To infect *T. confusum*, isolate and starve selected beetles for about 2 weeks. The eggs of *H. nana* are quite fragile and care must be taken to avoid excessive centrifugation speeds, undue exposure to concentrated salt solution, and desiccation. Concentrate the ova according to the Willis-Malloy brine flotation technique reducing centrifuging speed to 1000 rpm. Remove concentrated ova from brine solution after 10 minutes of contact between the coverslip and the meniscus. Wash the ova off the coverslips into a centrifuge tube and wash ova 4 or 5 times to remove salt. Transfer ova to a filter paper disc 1-1½ inches in diameter. Place the paper containing the ova on a large piece of filter paper and move it about to several spots to draw off excess moisture. Removal of moisture is especially important with *Tribolium* as they are easily caught up in any sticky substrate. To avoid drying out the delicate ova of *H. nana*, moisten a small piece of filter paper and adhere it beneath the dish cover to keep the interior slightly moist. Experience will indicate the balance between the needs of the beetle for a fairly dry bottom and that of the eggs for an atmosphere moist enough to prevent desiccation. Gravid proglottids of adult *H. nana* spread on a filter paper and well macerated can be used instead of the brine-concentrated eggs.

After 5-10 days of exposure of ova to the beetles, fill the dish with bran. These beetles can be retained for as long as 3 months from date of initial exposure to the eggs. However, if the beetles are to be kept for that length of time, infect a number considerably in excess of the number actually required, as a 50-percent loss is to be expected after 2 months and 70-90 percent after 3 months. Uninfected beetles live longer than infected ones; lighter infections will be found among the longer-lived survivors.

The cysticercoids of *H. nana* in either host beetle are infectious after 14 days and remain viable for the life of the beetle. They do not encyst in the intermediate host but remain free in the hemocoel or lightly attached to the gut or Malpighian tubules. *Tribolium* can be opened up in a drop of saline on a slide

and the cysticercoids teased out. The larger *Tenebrio* is best examined after clipping off the elytra and legs and tearing apart the abdomen in a watch glass containing several drops of physiological saline. If the cysticercoids are not to be retained for more than 3 or 4 hours before passage, tap water may be substituted.

Mice or rats should be infected with counted doses of cysticercoids by pipette rather than by feeding whole beetles. Forty to 50 cysticercoids per rat and 20-30 per mouse afford good infection levels. *H. nana* infections are self-limiting though reinfection via coprophagy is common.

### **Trichinella Spiralis**

Any of the commonly utilized laboratory animals and a wide range of mammals can be infected with the pork worm, *T. spiralis*. The animal most often utilized is the laboratory rat. All stages in the life cycle can be easily demonstrated by feeding bits of meat containing the encysted larvae to a number of animals simultaneously and then sacrificing them and examining tissues and organs for parasites at varying intervals following initial feeding of infected meat.

To pass the infection to rats, place them in clean cages without food, water, or litter, and starve them for several hours. Cut meat containing the encysted trichinellids into cubes about ¼ inch in diameter and distribute to clean Petri dishes in several gram quantities. Muscle tissue from the tongue, diaphragm, intercostal muscles, and masseters will contain the largest numbers of encysted larval worms. Introduce the dishes into the clean cages and observe the animals until all have eaten the meat. Stages of the cycle which can be demonstrated in the host animals include adult males and females in the intestinal tract, motile larvae in the circulating blood, larvae in the process of invading striated muscle, and larvae encapsulated in cysts in striated muscle tissue.

Following feeding of meat containing the infective larvae, the first stage which can be recovered from the host is the adult worm in

the lumen of the small intestine. Seventy-two hours after an animal has been infected, sacrifice the animal, open the abdominal cavity, and tie off the intestine at the following points: one tie at the rectal end, one tie just below the stomach, and two ties about  $\frac{1}{4}$  inch apart at the point of juncture of the caecum and small intestine. Sever the intestine between the ties at the caecum and above the tie adjacent to the stomach. Dissect out and transfer the intestine to saline in a Petri dish and with sharp pointed scissors, slit the intestine open throughout its length. With the gut immersed in saline, scrape the mucosa with a scalpel and discard the gut. Search the contents of the Petri dish under a stereoscopic dissecting microscope for the adult worms. Males are small, about 1.5 mm in length and bear at the caudal end two tongue-like appendages, and lack a spicule. The females are about two and one-half times as large and bear a genital orifice near the head end. At the end of 62 hours following infection, fertilization, which takes place only once, has occurred. Most of the males are eliminated from the intestinal tract within the first week following infection of the animal.

Larvae in the circulating blood are most readily recovered during the second week following infection. They may be found in the blood beyond the end of the third week after infection, but in considerably smaller numbers. To obtain larvae anesthetize an infected animal at any time during the second week of infection. Draw blood from the heart, transfer the blood to a centrifuge tube, add 9 ml of distilled water for each ml of blood to lake out red cells, and centrifuge at 500 rpm for 5 minutes. With a capillary pipette, draw off a few drops of the sediment and transfer to a drop of normal saline on a slide. Cover-slip the preparation and examine under low power for motile larvae.

To observe larvae in the process of encystment, sacrifice an animal any time between 15 and 25 days following infection. Remove a portion of diaphragm muscle and compress it between two slides, securing the slides firmly together with rubber bands or string. Examine the preparation under low power for migrating and encysting larvae. For recovery of encysted larvae, sacrifice an infected animal 6 weeks after infection or any time thereafter. The method of examination is the same as that described above for encysting worms.

## Appendix 3

### GLOSSARY OF TERMS

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*Anthelminthic:* A drug administered for the purpose of causing the death of intestinal worms with resultant dislodgement and elimination from the intestinal tract.

*Arthropods:* Animals belonging to the phylum Arthropoda, characterized chiefly by their many-jointed bodies. Insects comprise the principal members of this phylum which are of medical importance.

*Axostyle:* Rod-like organelles which function as internal structural support in certain protozoans, characteristically in the intestinal and vaginal flagellates.

*Biological Vector:* An arthropod vector in whose body the infecting organism develops before becoming infective to the recipient individual.

*Blepharoplast:* A minute oval or round granule forming a part of the complex known as the kinetoplast in blood and tissue flagellates belonging to the genera *Trypanosoma* and *Leishmania*.

*Cestode:* A common name applied to the tapeworms as a group.

*Charcot-Leydon Crystals:* Crystals which are greatly flattened in the longitudinal axis and terminate in needle-like points at their opposite poles. They are quite consistently observed in stools of individuals with amoebic dysentery.

*Chromatin:* The more readily stainable portion of a cell nucleus which displays a characteristic pattern useful in species identification.

*Chromatoid:* Variously shaped aggregations of material which stain deeply with hematoxylin, contained within the precystic and cystic stages of certain species of parasitic amoebae.

*Cilia:* Minute lash-like structures which serve as organelles of locomotion in protozoans belonging to the class *Ciliata*.

*Ciliate:* A general term applied to protozoans of the class *Ciliata*, characterized by the presence of numerous fine hair-like fibrils on the surface of the body which serve as organelles of locomotion.

*Cyst:* A sac-like structure the outer covering of which consists of a protective layer which envelops protoplasm of protozoans and enables them to survive under adverse environmental conditions.

*Cysticercosis:* The term applied to a disease in which the developmental larval stage of a tapeworm invades body tissue.

*Cysticercus:* A larval form of the tapeworm in which a single scolex is enclosed in a bladder-like cyst.

*Cytoplasm:* The protoplasm of the cell other than that of the cell nucleus.

*Cytostome:* An opening within the outer wall of certain species of highly developed protozoans which serves as a primitive mouth through which solid food or waste material passes in or out of the cell.

*Diurnal:* Activity occurring during both day-light and darkness.

*Echinococcosis:* The term applied to the disease which results from infection with the parasite *Echinococcus granulosus*.

*Ectoplasm:* The outer clear zone of cytoplasm on the immediate margin of the cell.

*Elephantiasis:* A disease caused by infection with *Wuchereria* sp., characterized by inflammation and enlargement of various parts of the body, most commonly the mammary glands, scrotum, and lower extremities.

*Embryophore:* A second egg shell formed by the embryo after loss of the first as seen in the eggs of *Taenia* sp. as they are found in the feces.

*Endoplasm:* The inner granular zone of cytoplasm within the cell.

**Fibrils:** Minute filaments which serve as organelles of locomotion in certain species of protozoans.

**Flagellate:** A general term applied to protozoans of the class *Flagellata* characterized by the presence of whip-like fibrils (flagella) which serve as organelles of locomotion.

**Gamete:** The sexual cell which is the end product of gametogeny in the life cycle of the malaria parasite. In a process comparable to that of fertilization in higher forms, the macro- and microgamete combine to produce the zygote within the body of the mosquito.

**Glycogen:** The chief carbohydrate form in which food is stored in the animal body. In protozoans glycogen stains very poorly with hematoxylin, and in stained preparations the poorly stained aggregations of glycogen are termed "glycogen vacuoles".

**Helminth:** A general term applicable to the various species of worms which may be parasitic in man.

**Hemozoin:** The pigment found within malaria parasites. Also the pigment deposited in body tissue as a result of the rupture of infected red cells at the completion of the schizogenous cycle of the malaria parasite.

**Hermaphroditic:** Possessing male and female reproductive organs in the same individual.

**Hexacanth:** The six-hooked embryo of certain species of tapeworms which is liberated from the egg at the time it hatches.

**Hyaline:** Glassy and transparent, or nearly so.

**Hydatid:** The cyst stage of an embryonic tapeworm in which the cyst contains daughter cysts, each of which contains many scolices.

**Karyosome:** One of the spherical masses of chromatin in the nucleus of a cell, generally situated at or near the center of the nucleus and more deeply staining with hematoxylin than the remaining nuclear chromatin.

**Leishmaniasis:** Any of three diseases characterized by the infection of the skin or internal organs with Leishman-Donovan bodies.

**Leishman-Donovan Body:** A small oval-shaped nucleated organism which is the

causative agent of all three forms of human leishmaniasis.

**Leptomonad:** The simple flagellate developmental stage of members of the genera *Trypanosoma* and *Leishmania*.

**Mechanical Vector:** An arthropod vector which transmits an infective organism from one host to another but which is not essential to the life cycle of the parasite.

**Merozoite:** Asexual forms in the developmental cycle of the malaria parasite which are liberated into the bloodstream when the merocyte reaches maturity.

**Metacercaria:** The encysted resting stage of a trematode either within the tissues of a crustacean or fish, or upon the surface of aquatic or semiaquatic vegetation.

**Miracidium:** The free-swimming larva liberated into the water from the egg of a blood fluke at the time the egg hatches.

**Nematode:** A general term applicable to all species of roundworms.

**Nocturnal:** Activity limited to the hours of darkness.

**Nucleus:** A spherical body within a cell which forms the essential and vital part that controls the cell's activities. It is distinguished from the remainder of the cell by its denser structure and consistent organization of chromatin material.

**Onchocerciasis:** A term applied to the disease which results from infection with the parasite *Onchocerca volvulus*.

**Oocyst:** The swollen sac-like structure which develops in the stomach wall of the mosquito as a result of invasion by the zygote. When mature, it gives rise to the sporozoites.

**Operculum:** The cap which covers the opening through which embryo of certain species of flukes and tapeworms escape from the eggs at the time of hatching.

**Ova:** The eggs of parasites containing the nutritive substances and germinal elements which give rise to a developmental stage in the life cycle of the parasite.

**Paragonimiasis:** The term applied to the disease which results from infection with the parasite *Paragonimus westermani*.

*Proglottids*: Individual divisions of the chain of segment-like structures which make up the body of tapeworms, exclusive of the head and neck.

*Protozoans*: Single-celled animals characterized by the fact that the body is composed of one or more nuclei surrounded by cytoplasm and contained within a limiting cell membrane.

*Pseudopod*: A temporary protrusion of the outer margin of the cell wall of an amoeba serving for purposes of locomotion and feeding.

*Rhabitiform*: The first stage larva liberated from the ova of a nematode worm.

*Rostellum*: The hook-bearing portion of the head of certain parasitic intestinal worms.

*Schistosome*: The general term applied to the blood flukes.

*Schistosomiasis*: A term designating the disease produced by infection with any of the three species of blood flukes which may parasitize man.

*Schizogeny*: The asexual cycle of sporozoa; particularly the asexual cycle of the malarial parasite in the blood corpuscles of man.

*Scolex*: The attachment end of a tapeworm consisting of the head and neck.

*Sparganosis*: The term applied to the disease produced by the migration of larval tape-worms within body tissues.

*Sporocyst*: The cyst or sac which forms within a snail as a result of entry of the miracidial stage of a fluke.

*Sporozoite*: The infective stage of the malarial parasite which migrates to the salivary gland of the mosquito.

*Strobila*: The entire adult tapeworm including the head, neck, and chain of proglottids.

*Trematode*: A common name applied to the flukes as a group.

*Trichinosis*: A disease caused by infection with the "porkworm", *Trichinella spiralis*.

*Trophozoite*: The active vegetative feeding motile stage of a protozoan.

*Trypanosome*: A blood and tissue flagellate of the genus *Trypanosoma*.

*Vacuole*: A term applied to various small aggregations of material (generally food) which float about in the cytoplasm within protozoan cells.

*Vector*: A carrier, especially the animal (usually an arthropod) which transfers an infective agent from one host to another.

*Viable*: In living condition.

*Volutin*: Chromatin-like bodies found within the cytoplasm of trypanosomes.

*Xenodiagnosis*: Diagnosis accomplished by allowing a known natural uninfected vector to feed upon a suspected infected individual for the purpose of attempting to recover the organism from the vector.

*Zygote*: The cell resulting from the fusion of the two gametes.



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